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Lysosomes, weapons or shield in gentamicin-induced apoptosis?

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« Le système général des sciences et des arts est une espèce de labyrinthe, de chemin tortueux, où l'esprit s'engage sans trop connaître la route qu'il doit tenir. »

Jean le Rond d'Alembert

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ABBREVIATION LIST:

AIF: Apoptosis inducing factor
ANT: Adenine nucleotide translocase
Apaf: Apoptotic protease activating factor
ARC: Apoptosis regulator with a CARD domain
BH: Bcl-2 homology
CAD: Caspase activated DNAse
CARD: Caspase activation recruitment domain
CK: Creatine kinase
CyD: Cyclophilin D
DED: Death effector domain
DISC: Death inducing signalling complex
EDP-I: Energy-dependent phase I
EDP-II: Energy-dependent phase II
EGF: Epidermal growth factor
FADD: Fas Associated Death Domain
FLIP: FLICE-inhibitory protein
Hsp: Heat shock protein
HGF: Hepatocyte growth factor
HK: Hexokinase
IAP: Inhibitor of apoptosis protein
IGF-1: Insulin-like growth factor 1
JNK: c-jun N-terminal kinase
LDH: Lactate deshydrogenase
MAPK: Mitogen-activated protein kinase
NFκB: Nuclear factor binding to the Igκ promoter in B cells
PARP: Poly (ADP-ribose) polymerase
PDI: Protein disulfide isomerase
PI3K: Phosphatidyl-inositol-3' kinase
PKC: Protein kinase C
PTP: Permeability transition pore
ROS: reactive oxygen species
SIMP: Soluble intermembrane mitochondrial protein
SODD: Silencer of death domain
TNF: Tumor necrosis factor
TRAIL: TNF-related apoptosis inducing ligand
TRADD: TNF receptor associated protein with a death domain
UPP: Ubiquitin proteasome pathway
UPR: Unfolded protein response
VDAC: Voltage-dependent anion channel

CONTENT

CHAPITRE I: INTRODUCTION : 11

1. AMINOGLYCOSIDES ANTIBIOTICS: 12
1.1. CHEMICAL STRUCTURE OF AMINOGLYCOSIDES 13
1.2. ANTIBACTERIAL ACTIVITY OF THE AMINOGLYCOSIDES 17
1.2.1. Uptake of aminoglycosides 17
1.2.2. Mechanism of action 18
1.3. TOXICITY INDUCED BY AMINOGLYCOSIDES 23
2. AMINOGLYCOSIDS NEPHROTOXICITY 25
2.1. EPIDEMIOLOGY : 25
2.2. CLINICAL FEATURES 25
2.3. RISK FACTORS OF AMINOGLYCOSIDE NEPHROTOXICITY 26
2.3.1. Patient-related factors: 26
2.3.2. Drug-related-factors 28
2.4. HANDLING OF AMINOGLYCOSIDES BY THE KIDNEY 30
2.4.1. Mechanism of cortical uptake: 31
2.4.2. Intracellular handling : 31
2.4.3. Mechanisms of toxicity 32
2.5. MEANS OF PROTECTION: 37
2.5.1. Clinical approaches: 38
2.5.2. Experimental approaches: 41
2.6. CONCLUDING REMARKS 44
3. CELL DEATH: APOPTOSIS 45
3.1. KEY COMPONENT OF THE APOPTOTIC SIGNALLING 46
3.1.1. Caspases 46
3.1.2. Proteins of the Bcl-2 family 48
3.2. MOLECULAR PATHWAY LEADING TO APOPTOSIS 55
3.2.1. Receptor-mediated (extrinsic) pathway 55
3.2.2. Organelle-mediated (intrinsic) pathway 57
4. APOPTOSIS IN THE KIDNEY 72
4.1. APOPTOSIS DURING KIDNEY DEVELOPMENT 72
4.2. APOPTOSIS INDUCED BY NEPHROTOXIC AGENTS 74
4.3. STRATEGIES TO DECREASE CELL DEATH-INDUCED BY NEPHROTOXIC AGENTS 80
4.3.1. Decreasing drug accumulation in the kidney 81
4.3.2. Regulating the molecular pathways and pro- or anti-apoptotic factors 81
4.3.3. Stimulating the survival pathway and regulating the cell cycle 84

4.3.4. Gene therapy 86

CHAPITRE II: AIM OF THE STUDY AND ELEMENTS THAT HAVE LED TO THIS WORK89

CHAPITRE III: RESULTS: LYSOSOMES, WEAPONS OR SHIELD IN GENTAMICIN-INDUCED APOPTOSIS. 95

1. GENTAMICIN-INDUCED APOPTOSIS IN LLC-PK1 CELLS: INVOLVEMENT OF LYSOSOMES AND MITOCHONDRIA 96

2. INVOLVEMENTS OF CATHEPSINS B AND D IN GENTAMICIN-INDUCED APOPTOSIS? 111

2.1. INTRODUCTION 111

2.2. RESULTS 113

2.2.1. Protective effect of pepstatin A but not leupeptin 113

2.2.2. Cathepsin D was not found released from the lysosomes to the cytosolic compartment 115

2.3. ADDITIONAL MATERIALS 116

2.4. CONCLUSION: 117

2.5. DISCUSSION: 117

3. GENTAMICIN CAUSES APOPTOSIS AT LOW CONCENTRATIONS IN RENAL LLC-PK_1 CELLS SUBJECTED TO ELECTROPORATION 120

CHAPITRE IV: CONCLUSIONS AND GENERAL DISCUSSION : 131

1. MAIN FINDINGS OF THIS WORK 132

2. MAIN ISSUES ARISING FROM OUR OBSERVATIONS 135

2.1. ARE LLC-PK1 CELLS AN APPROPRIATE CELLULAR MODEL? 135

2.2. COULD LYSOSOMAL DESTABILIZATION BE RESPONSIBLE OF GENTAMICIN DELIVERY INTO THE CYTOSOL OF INCUBATED CELLS? 136

2.3. WHAT CAN WE CONCLUDE ABOUT THE INVOLVEMENT OF LYSOSOMAL CATHEPSINS IN GENTAMICIN-INDUCED APOPTOSIS? 139

2.4. WHAT IS THE RELEVANCE OF THE AMOUNT DEFINED BY ELECTROPORATION AS "APOPTOGENIC" WITH IN VITRO OBSERVATIONS IN LLC-PK1 CELLS? 141

2.5. HOW CYTOSOLIC GENTAMICIN CAN INDUCE CELL DEATH? 143

2.5.1. Gentamicin acts on its primary target: 18S rRNA (the eukaryotic homologue of the 16S rRNA):143

2.5.2. Gentamicin could act by altering the 26S proteasome. 149

2.5.3. Gentamicin could directly disturb mitochondria 151

2.6. WHAT ABOUT THE EVOLUTION OF OUR COMPREHENSION OF AMINOGLYCOSIDES NEPHROTOXICITY?152

CHAPITRE V: PERSPECTIVES 159

1. SHORT TERM PERSPECTIVES. 160

1.1. IMPORTANCE OF THE LYSOSOMAL DESTABILIZATION 160

1.2. IDENTIFICATION OF THE CYTOSOLIC TARGET OF GENTAMICIN RESPONSIBLE FOR APOPTOSIS INDUCTION. 161

1.2.1. Production of misfolded protein and inhibition of protein synthesis by gentamicin. 161

1.2.2. Importance of the inhibition of the proteasome in gentamicin-induced apoptosis: 162

1.2.3. Importance of mitochondrial targeting of gentamicin in the induction of apoptosis. 163

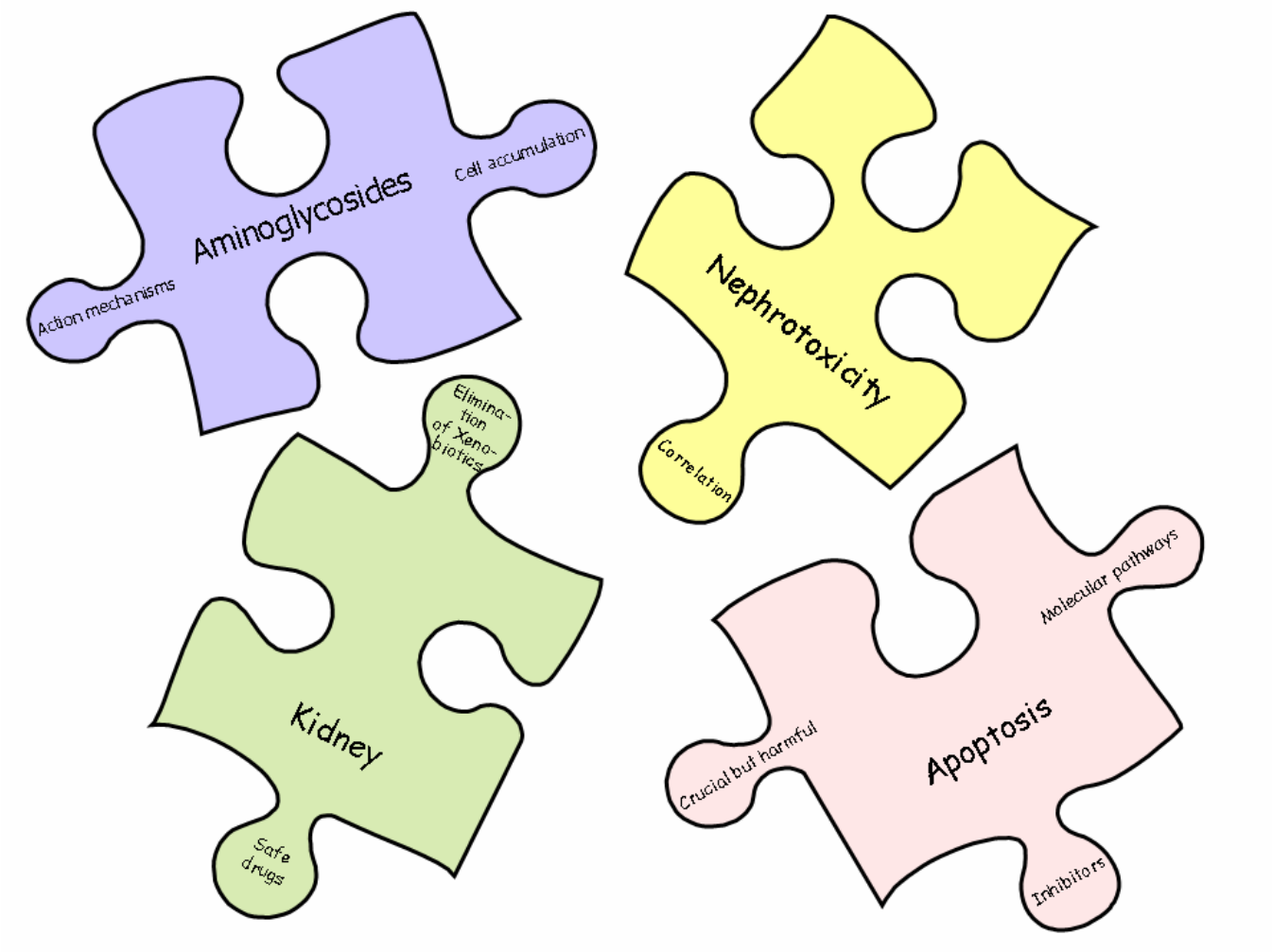
2. LONG TERM PERSPECTIVES: 164

CHAPITRE VI: REFERENCES 167

CONTENT

Chapitre I: INTRODUCTION :

When drugs create « Disorder »…



What about the aminoglycosides….

1. AMINOGLYCOSIDES ANTIBIOTICS:

Following the discovery of the first antibiotic penicillin, active against Gram-positive bacteria, Waksman isolated in 1946 the first anti-tuberculosis drug, streptomycin (Waksman SA and Schatz AI, 1946). This compound gave rise to a new class of antibacterial substances called aminoglycosides. With interesting antibacterial properties, aminoglycosides completed the antibacterial drug arsenal thanks to their activity against Gram-negative bacteria. Aminoglycosides are naturally derived antimicrobial substances. Streptomycin was produced by a *Streptomyces griseus* organism (Waksman SA and Schatz AI, 1946), together with its closely related derivative dihydrostreptomycin, they have been widely used from the late 40's through the mid 60's to treat Gram negative infections and tuberculosis. Emergence of resistance and the desire to enlarge the spectrum towards "difficult to treat bacteria" such as *Pseudomonas aeruginosa*, led to the development and introduction of kanamycins (represented mostly by kanamycin A and tobramycin, and, in some countries, by dibekacin), sisomicin, and gentamicin. Gentamicin is a fermentation product isolated from actinomycete *Micromonospora purpurea* (Weinstein M.J. *et al.*, 1963) and consists in a mixture of three major components referred to as gentamicin C_1, C_{1a}, and C_2. These were extensively used up to the mid 80's. Further development of resistance to these naturally-occurring molecules eventually triggered the design of semi-synthetic derivatives (amikacin [from kanamycin A], netilmicin [from sisomicin], isepamicin [gentamicin B], and arbekacin [from dibekacin] that were made to resist to bacterial inactivating enzymes responsible for this resistance (Mingeot-Leclercq *et al.*, 1999). All along these developments, ototoxicity and nephrotoxicity remained, however, of concern to clinicians, and resulted in a marked limitation in the use of these otherwise potent and life-saving antibiotics. The industry largely if not entirely quitted this field of research from the late 80's until the late 90's. During this period, research on aminoglycosides (mainly conducted by university groups) was principally aimed at deciphering the mechanism

underlying toxicity but also at reducing their toxicity which was the so far only means of protecting patients.

However, from the late 90's until now, critical advances have been made about the mechanism of action and in particular at understanding of the exact interaction of aminoglycosides with their target: the 16S ribosomal RNA A-site. Consequently, these numerous discoveries have revived the race to the development of new "aminoglycosides". Indeed, various research groups from the academy (Haddad *et al.*, 2002; Russell *et al.*, 2003) or from the Industry (Fridman *et al.*, 2003; Ding *et al.*, 2001; Ding *et al.*, 2003) (Yao *et al.*, 2004; Vourloumis *et al.*, 2002; Simonsen *et al.*, 2002; Vourloumis *et al.*, 2003; Simonsen *et al.*, 2003; Barluenga *et al.*, 2004) have published new antimicrobial decoding-site ligands based on aminoglycosides derivates and mimetics that were, in part, designed using structural information of the bacterial RNA target.

If Industries have now a well-validated target for the development of new aminoglycosides, the exact mechanisms by which these molecules are toxic remain elusive, and nevertheless certainly of critical importance for the development of new compounds. In this context, the demonstration that aminoglycosides are able to induce renal proximal tubular cell death by apoptosis *in vivo* but also *in vitro* by the use of doses relevant to clinical situations, have brought new perspectives (El Mouedden *et al.*, 2000b; El Mouedden *et al.*, 2000b). However, some questions remained unresolved… Which is the exact mechanism by which aminoglycosides induce apoptosis? As aminoglycosides are mainly localized insides the lysosomes of the renal proximal tubular cells, are they able to induce death by altering lysosomal functions? Or are they toxic by having access to other cellular constituents?

1.1. Chemical structure of aminoglycosides

Aminoglycoside antibiotics are polar, cationic molecules at physiologic pH, this particularity is critical not only for their antibacterial activity but also for their toxicity. Both of these points will be developed later.

All aminoglycosides have an essential six-membered ring with amino-group substituents called aminocyclitol ring (represented in figure 1 by the central ring). This ring is linked with two or more amino-containing or non-amino-containing sugars. For the majority of clinically useful compounds, the aminocyclitol is 2-deoxystreptamine ring, and it can be disubstituted in position 4 and 5 (as it is the case for paromomycin and neomycin) or in position 4 and 6 (as it is the case for gentamicin or amikacin) (see figure 1). The accepted nomenclature usually used (decribed by Nagabushushan (Nagabhushan TL *et al.*, 1982) refers to ring I as the primed ring and corresponds to the aminosugar at position 4 of the deoxystreptamine ring. Ring II is unprimed and corresponds to the central aminocyclitol, while ring III is referred to as the doubly primed ring and has the substituent in position 5 or 6 of the deoxystreptamine ring. Ring IV (triply primed numbering) correspond to any additional ring attached to ring III (see figure 1). It has also to be mentioned that other atipycal aminoglycosides (according to the above description) have been used in clinical settings: these are streptomycin (possesses a streptidine central ring) and spectinomycin (which consists of three fused ring).

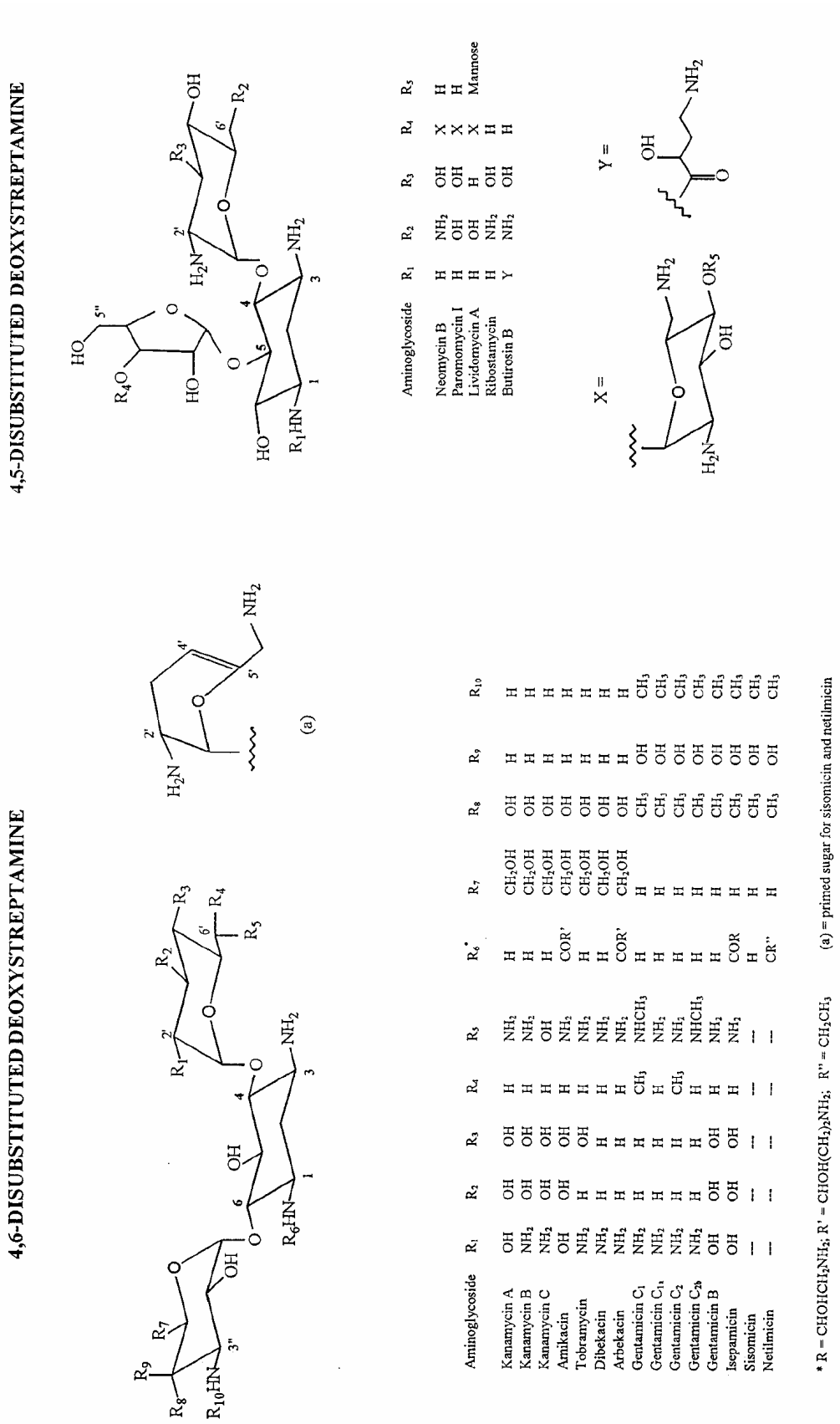
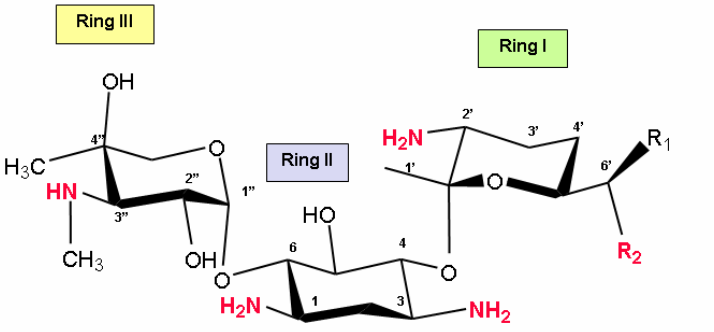


***Figure 1**: Structures of typical aminoglycosides containing a 2-deoxystreptamine central ring. From (Mingeot-Leclercq and Tulkens, 1999)*

Gentamicin, one of the most used aminoglycosides in clinical practice, possesse a central aminocyclitol ring which is 2-deoxystreptamine (Ring II, figure 2). This ring is substituted at position 4 and 6 with two sugars by glycosidic bond (Rings I and III, figure 2). Gentamicin is not a single compound but a mixture of three major components, gentamicin C_1, C_{1a} and C_2. They differ in the degree of methylation in the 2-amino-hexoses (purpurosamine or ring I) ring. Gentamicin C_{1a} lacks the methyl group in 6' position of ring I, while gentamicin C_1 and C_2 have this methyl group in 6' position. Gentamicin C_1 is also N-methylated in this position, while C_{1a} and C_2 have free amines instead (see figure 2: the different constituents of the three major compounds of gentamicin are specified in the table below).

Gentamicin has a molecular weight of 464 daltons and its overall pKa value is about 8.4, individual pKa values of the amino groups range from 5.5 to 9 (Claes P.J. *et al.*, 1977) and is positively charged in acidic environment as it is the case inside the lysosomes, their major site of localisation (pH around 5.4) (Ohkuma and Poole, 1978).



	R_1	R_2
Gentamicin C_1	CH_3	$NHCH_3$
Gentamicin C_{1a}	H	NH_2
Gentamicin C_2	CH_3	NH_2

Figure 2: *Structure of gentamicin. Commercial gentamicin used for this study is a mixture of gentamicin C1, C1a, and C2 compounds in the following proportion 30%, 20% and 50% respectively (Geomycine® Shering-Plough).*

The amino-groups, positively charged at physiological pH, are not only responsible for their uptake by the bacteria but also for their mechanism of action. How can these agents be taken up by bacteria and how are their able to act inside the bacteria?

1.2. Antibacterial activity of the aminoglycosides

1.2.1. Uptake of aminoglycosides

The penetration of aminoglycosides inside bacteria is divided into three consecutives steps.

The first step involves an electrostatical, rapid and energy-independent binding of positively charged substituents of aminoglycosides to negatively charged moieties of lipopolysaccharides, polar heads of acidic phospholipids, and anionic outer membrane proteins in gram negative bacteria and to negatively-charged phospholipids and teichoic acid in gram-positive microorganisms (Laurent *et al.*, 1990; Taber *et al.*, 1987). In gram negative bacteria, their binding displaces the Ca^{++} and Mg^{++} that normally link adjacent lipopolysaccharide molecules (Hancock, 1984); (Peterson *et al.*, 1985), resulting in a rearrangement of these molecules and the formation of transient hole in the cell wall leading to cell permability (Martin and Beveridge, 1986). This binding results in the accumulation of aminoglycosides inside the periplasmic space (Humbert and Altendorf, 1989).

After ionic binding, the uptake can be divided into two phases named EDP-I, a slow phase and EDP-II, a subsequent rapid phase consisting of the fixation of aminoglycoside to the 30S subunit ribosome (Bryan and Kwan, 1983; Taber *et al.*, 1987; Hancock and Bellido, 1992; Hancock, 1981a; Hancock, 1981b). Both EDP-I and EDP-II phases are energy-dependent. Uptake of aminoglycoside during phase I require a threshold transmembrane potential generated by a membrane-bound respiratory chain. That is why microorganisms with deficient electron transport system, such as anaerobes, are intrinsically resistant to aminoglycosides. It is though that during phase I, only a small quantity of

antibiotic cross the cytoplasmic membrane (Bryan and Van Den Elzen, 1977; Damper and Epstein, 1981). The binding of incoming antibiotic to the ribosome results in misreading of mRNA and production of misfolded proteins. Some of these proteins are incorporated in the cytoplasmic membrane, contributing to the loss of membrane integrity. This in turn triggers a cascade of events called EDP-II. During this phase, additional amounts of aminoglycoside are transported and they accumulate rapidly in the cytoplasm.

1.2.2. **Mechanism of action**

Aminoglycosides belong to the family of antibiotics acting by **impairing the protein synthesis** (Davies *et al.*, 1965; Davies and Davis, 1968), with tetracyclines, macrolides, streptogramins, chloramphenicol and oxazolidinones. Most aminoglycosides and tetracyclines impair protein synthesis by interaction with the **16S small ribosome subunit** (precursor of the functional 30S particle) whereas the other cited antibiotics interact with the 32S (precursor of the functional 50S particle) large ribosomal subunit.

The high resolution crystal structures of the ***30S ribosomal particle*** free or bound with antibiotics (in particular with paromomycin) (Carter *et al.*, 2000) and with various tRNA-mRNA analogues have offered the ultimate illustration of aminoglycosides mechanism of action (Wimberly *et al.*, 2000; Ogle *et al.*, 2001; Ogle *et al.*, 2002). Inside the 30S particle, paromomycin has only one specific binding site, the **A-site** (see figure 3).

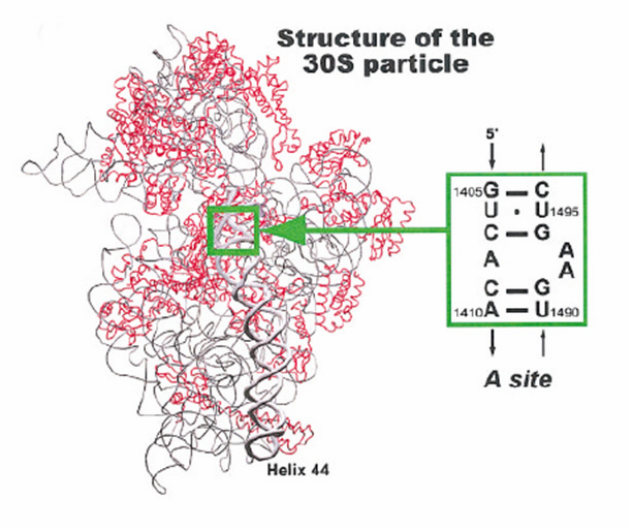


Figure 3: *Crystal structure of the 30S particle. View of the 50S-facing side, with the 16S rRNA in grey and the proteins in red. The A-Site is located at the foot of helix 44. The box on the right shows the A-Site structure according to the E. coli numbering. From (Vicens and Westhof, 2003a).*

This site is located at the foot of helix 44, the longest helix of the 30S particle on the face that contacts the 50S particle. In the absence of any aminoglycoside, the A-site is formed by three Watson-Crick G=C pairs, one U.U pair, and an internal loop made of three adenines (1408, 1492 and 1493 according *E. coli* numbering) in which A1492 and A1493 fold back within helix. When paromomycin is complexed with this 30S particle, it binds to the deep groove of the A-site by inserting ring I inside the helix, this puckered ring stacks against G1491 and forms two direct hydrogen bonds to A1408. The main effect of this insertion is to force adenine 1492 and 1493 to bulge out from the helix (see figure 4). But what is the physiological consequence of this bulging out of both adenines?

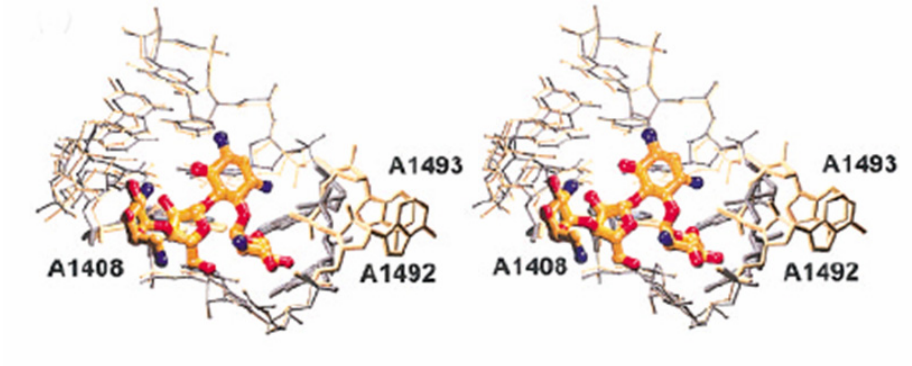


Figure 4: *Superimposition (based on the sugar-phosphate backbone atoms) of the A-site free (grey) and bound to paromomycin (gold) inside the 30S particle. From (Vicens and Westhof, 2003a).*

During decoding step of protein synthesis, the A-site changes its conformation from an "off" conformation (wich can be found in two states: the first one where A1492 and A1493 are folded in the shallow groove of the A-site and the second one where only one of these adenines is found inside the A-site (Shandrick *et al.*, 2004; Abelian *et al.*, 2004); (Francois *et al.*, 2005) to an "on" conformation (A1492 and A1493 fully bulge out from the A-site) (Ogle *et al.*, 2002; Wimberly *et al.*, 2000; Ogle *et al.*, 2001). This conformational change is necessary to allow A1492 and A1493 to specifically interact with the first two of the three base pairs formed by the cognate codon-anticodon interaction (Ogle 2001 897). It also provokes the transition of the ribosome from an open to closed form that is stabilized by contacts involving the cognate tRNA and the ribosome (Ogle *et al.*, 2002; Ogle *et al.*, 2003). **Aminoglycosides lock the A-site in the open conformation** and by doing so they also pay for a part of the energetic cost associated with the tRNA-dependent ribosome closure (Ogle *et al.*, 2002; Ogle *et al.*, 2003). As the consequence, the **ribosome has lost its ability to discriminate cognate versus non-cognate tRNA-mRNA** associations (Ogle *et al.*, 2003; Ogle *et al.*, 2001)).

High resolution crystal structure of ***oligonucleotides containing the A-site*** complexed with different aminoglycoside has allowed precise identification of the **molecular binding characteristics of aminoglycosides** within the A-site of the 16S rRNA (Vicens and Westhof, 2001; Vicens and Westhof, 2002;

Francois *et al.*, 2005). This model has revealed some specific interactions, involving both electrostatic interactions and hydrogen bonding between the phosphate backbone of the RNA and the positively-charged amino groups of the aminoglycoside, some of them being conserved for most aminoglycosides and others being more specific to a given aminoglycosides. These observations have confirmed those described by crystallography study of the entire 30S particle for paromomycin and the previously described interactions (NMR, biochemical, mutagenesis studies) (Fourmy *et al.*, 1998; Purohit and Stern, 1994); (Yoshizawa *et al.*, 1998; Vicens and Westhof, 2002).

The insertion and interactions of **gentamicin C_{1a} with decoding A-site oligonucleotides** have been revealed recently by François and co-workers (Francois *et al.*, 2005). The interaction of gentamicin C_{1a} with the A-site is similar to that observed for other aminoglycosides, some of these interactions are indeed invariant intermolecular contacts between rings I and II: Ring I forms a pseudo-base pair with two direct H-bonds to the Watson-Crick sites of A1408. Nitrogen of the 2-deoxystreptamine makes some H-bond: N1 to 04(U1495), N3 to N7(G1494), O2P(G1494), O1P(A1493). They are shown in red in figure 5.

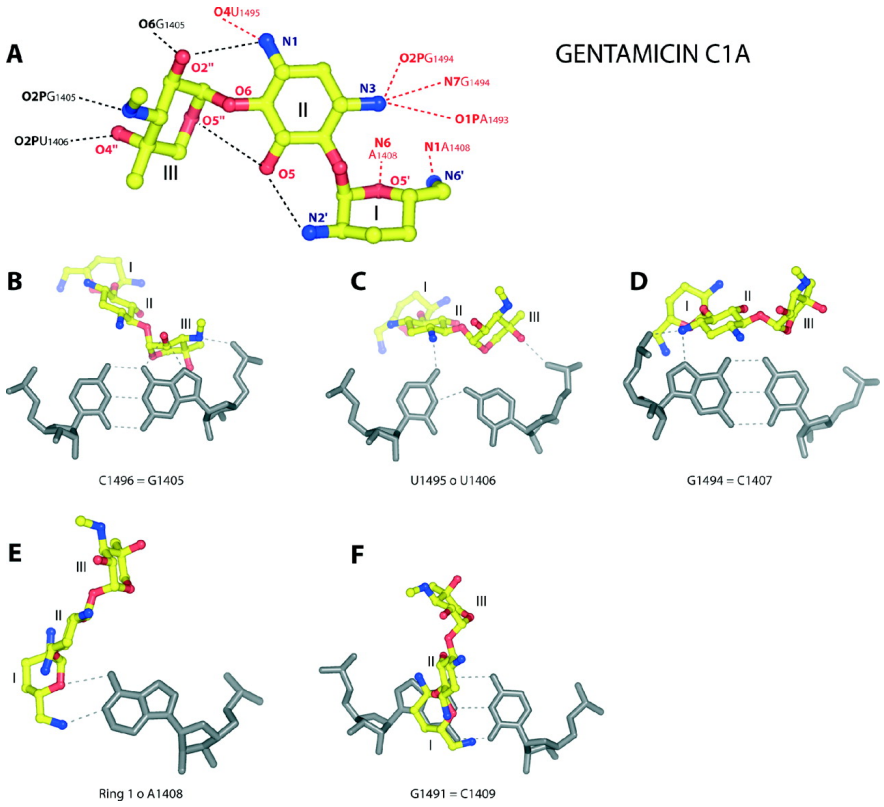


Figure 5: *Description of the contacts between gentamicin C1a and the RNA fragment corresponding to the A-site. (A) Structure adopted by gentamicin C1a molecule inside the A-site of the RNA fragment. The E. coli numbering is used for the RNA atoms. Hydrogen bonds are shown as dashed blue lines. Conserved contacts between gentamicin C1a and the A-site are shown in magenta.(B-F) Atomic details of the contacts involving each base pair of the minimal A-site interacting with gentamicin C1a. From (Francois et al., 2005).*

Additional interactions consist in the following and contribute to the stabilization of the complex: the U1495.U1406 base pair is bifurcated with a H-bond between O4'' and O2P(U1406), the intramolecular H-bond between N2' and O5 linking rings II to I, O2'' and N1 linking rings III to II and between O5 and O5'' linking rings I and III are direct bonds. In addition, O2'' makes direct bond with O6(G1405), O4'' with O2P(U1406) and finally N3'' with O2P(G1405).

However, while aminoglycosides binding to the prokaryotic A-site RNA have shown some specificity, other studies suggest that aminoglycosides binding to the A-site is not so specific. Indeed, it has been shown (1) that structurally diverse aminoglycosides can bind to the A-site with **relatively low affinity** ranging from 0.1 to 10 µM (Wong *et al.*, 1998) (Carter *et al.*, 2000; Ryu and Rando, 2001; Wang *et al.*, 1997), (2) that aminoglycosides can also bind the

eukaryotic decoding region construct with approximately the same affinities found with their prokaryotic counterpart, even though the two A-site structures are different (Ryu and Rando, 2001), (3) that aminoglycosides can bind in a saturable fashion to a wide-variety of RNA structures with similar µM affinities. Indeed, aminoglycosides have been shown to bind and interfere with the function of HIV TAR (Wang *et al.*, 1998b) and HIV RRE (regulatory RNA elements) (Zapp *et al.*, 1993), the hammerhead (Stage *et al.*, 1995) and hepatitis delta virus (Rogers *et al.*, 1996) ribozyme, self-splicing group-I introns (von Ahsen and Noller, 1993; Hoch *et al.*, 1998), bacterial RNasesP (Mikkelsen *et al.*, 1999), and tRNA (Kirk and Tor, 1999; Kotra *et al.*, 2000). But how aminoglycosides could interact with all these RNA structures? The probable explanation for that is the particular tri-dimensional structure of all these RNA molecules which have been shown to contain non-duplex structural elements generally either bulges or bubbles (Cho and Rando, 1999). As aminoglycosides are no rigid molecule, they are probably able to adapt conformationaly for their insertion inside these particular structures, the electrostatic and hydrogen bonding described between aminoglycosides and the A-site probably mainly contribute to the stabilization of the complex.

In conclusions, the numerous studies conducted to characterize the molecular interaction of aminoglycosides have opened very broad new perpectives, not only for the design of “new aminoglycosides” but also for the development of a new class of drugs, the small-molecule-RNA ligand with numerous possible applications (Sucheck and Wong, 2000; Mayer and James, 2005; Hermann, 2005).

1.3. Toxicity induced by aminoglycosides

If the antibacterial activity of the aminoglycosides is now well established, the limitation for such new drugs will reside also in their potential for toxicity. Indeed, all the aminoglycosides (exception made for spectinomycin), share a same potential for causing injury to the renal proximal convoluted tubules,

damage to the choclea and/or vestbular apparatus and neuromuscular blockade (Pittinger and Adamson, 1972; Swan, 1997; Brummett and Fox, 1989). As mentioned earlier the inherent toxicity of aminoglycoside is linked to their positive charge at physiological condition (Berdy J *et al.*, 1980). The **chochlear ototoxicity** and **nephrotoxicity** were encountered with frequencies reaching 62 and 50% respectively. Vestibular ototoxicity blockade is less frequent with value of 19% and neuromuscular blockade is rather rare side effect and occurred if injection is too rapid or if in presence of other toxic drugs.

Ototoxic hearing loss usually begins in high frequencies and is secondary to irreversible destruction of outer hair cells in the organ of Corti, predominantly at the basal turn of cochlea (Forge and Schacht, 2000). Aminoglycosides have been detected in the cochlea months after the last dose administration. This retention may account for delayed onset earing loss and prolonged susceptibility to noise-induced hearing loss, which is often observed for several months post therapy.

There is growing evidence that aminoglycosides induced hair cell loss results from apoptosis (Forge, 1985; Li *et al.*, 1995; Nakagawa *et al.*, 1998) and that hair cell death involves the activation of initiator caspase-9 and caspase-8 followed by effector caspase-3 (Cheng *et al.*, 2003; Cunningham *et al.*, 2002a). Recently, minocyclin known to inhibit mitochondrial-activation has been demonstrated protective for hair cells in neonatal cochlear culture (Corbacella *et al.*, 2004). All these observations suggest a mitochondrial-pathway. Other initiators of apoptosis have also been described, such as the generation of ROS by the formation of iron-gentamicin complex (Priuska and Schacht, 1997), (Priuska and Schacht, 1995; Sha and Schacht, 1999; Clerici and Yang, 1996) but also the activation of the c-Jun Kinase pathway (Pirvola *et al.*, 2000).

In the opposite to ototoxic reaction, nephrotoxicity of aminoglycosides is reversible and kidney recovers when the administration of drug is stopped. But what do we know about this nephrotoxicity?

2. AMINOGLYCOSIDES NEPHROTOXICITY

This section is a part of a book chapter in Toxicology of the Kidney (Servais H. *et al.*, 2005). It is presented with some important updates.

2.1. Epidemiology :

The reported incidence of aminoglycoside nephrotoxicity varies from 0-50% with most reports in the 5-25% range (Lane *et al.*, 1977; Lerner *et al.*, 1983; Bertino, Jr. *et al.*, 1993). This variability results from differences in definition of nephrotoxicity, nature and frequency of the criteria used to assess renal function and, perhaps most importantly, the clinical setting in which the drugs are used. The incidence in aged patients suffering from multi-system diseases and exposed to other potential nephrotoxins ranges as high as 35-50%, whereas this figure may be close to 0 in young healthy volunteers (Lane *et al.*, 1977; Appel, 1990). In prospective randomized studies with definitions of nephrotoxicity that reflect a substantive decrement of glomerular filtration rate in seriously ill patients, the reported incidence of nephrotoxicity varies between 5-10% of patient courses (Lane *et al.*, 1977; Smith *et al.*, 1977). In surveys of the etiology of acute renal failure in hospitalized patients, about half of the drug-induced cases of renal toxicity were attributable to aminoglycosides.

2.2. Clinical features

Nephrotoxicity induced by aminoglycosides manifests clinically as nonoliguric renal failure, with a slow rise in serum creatinine and a hypoosmolar urinary output developing after several day of treatment. The first signs of tubular dysfunctions or alterations are the release of brush border and lysosomal enzymes, decreased reabsorption of filtered proteins, wasting of K^+, Mg^{2+}, Ca^{2+} and glucose; phospholipiduria. Progression to dialysis-dependent oliguric-anuric renal failure is unusual unless other risk factors are present (Appel, 1990). The renal failure is generally reversible. In a few patients there has been documented recovery of renal function despite continued administration of the

aminoglycoside (Trollfors, 1983). Conversely, cases of fatal anuria have been reported. Occasionally, a Fanconi's syndrome (Casteels-Van Daele *et al.*, 1980) or a Bartter's-like syndrome (Landau and Kher, 1997) has been observed.

2.3. Risk factors of aminoglycoside nephrotoxicity

Based on clinical observations, there appear to be a variety of factors that predispose to the development of renal dysfunction with aminoglycoside therapy (Appel, 1990). It is important to distinguish between patient- and drug-related factors since this will guide the caregiver for taking the most appropriate course of action.

2.3.1. Patient-related factors:

A. Age:

The incidence of amikacin nephrotoxicity rises with advancing age from 7 % in patients under age of 30 years to 25 % in patients over 75. Two main mechanisms explain this situation. First, the number of active nephrons decreases with age leaving the patient with less reserve upon injury to a given number of them. Second, because of the falling renal function, dosages may often be excessive if only based on insensitive renal function tests such as the measurement of serum urea nitrogen or serum creatinine which rise significantly only when a large proportion of active nephrons is damaged (Moore *et al.*, 1984).

B. Pre-existing renal diseases:

In patients with pre-existing renal disease, as estimated by serum creatinine greater than 2 mg/dl, the study of Moore et al. (Moore *et al.*, 1984) found no increase in toxicity risk if the dose was carefully adjusted. Yet, pre-existing renal failure clearly exposes the patient to overdosing. In addition, the kidney from patients with pre-existing renal disease may have a decreased ability to recover from ischemic and/or toxic insults (Manian *et al.*, 1990; Beauchamp *et al.*, 1992).

C. Female or male gender:

Female gender was identified as a risk factor in one study but not confirmed in others (Kahlmeter and Dahlager, 1984; Moore *et al.*, 1984). Conversely, male gender was also reported in a retrospective analysis as a risk factor (Bertino, Jr. *et al.*, 1993). The matter is, therefore, unsettled (animal studies are of little value in this context since most are performed with rats, and we know that male laboratory rats tend to be spontaneously proteinuric, which in it-self may be a risk factor).

D. Volume depletion/hypotension:

Depletion of intra-vascular volume is an important risk factor for aminoglycoside-induced nephrotoxicity whether induced by sodium depletion, hypoalbuminemia, or diuretics, even when systemic acid-base and electrolyte/volume status are maintained (Gamba *et al.*, 1990). Hypokalemia and hypomagnesemia may be both predisposing risk factors or consequences of aminoglycoside-induced damage (Nanji and Denegri, 1984; Zaloga *et al.*, 1984).

E. Liver diseases:

Liver dysfunction was identified as a risk factor in the retrospective analysis of two large clinical trials and was then validated in two additional prospective trials (Lietman, 1988). This is particularly true of patients with biliary obstruction, cholangitis or both rather than other causes of liver disease such as alcoholic cirrhosis (Desai and Tsang, 1988). There is no simple explanation to this observation.

F. Sepsis:

Because of the unique role of aminoglycosides in treating patients with difficult gram-negative sepsis, the hemodynamic and metabolic perturbations of the sepsis syndrome often were associated with an increase in drug-induced nephrotoxicity. Acute or chronic endotoxemia amplifies the nephrotoxic

potential and renal uptake of gentamicin in rats (Ngeleka *et al.*, 1990; Auclair *et al.*, 1990; Tardif *et al.*, 1990). We, however, lack of definitive studies in humans (cases of renal failure that could be associated with the administration of batches of endotoxin-contaminated gentamicin have, however, been observed). The mechanism can be complex, but is thought to result from the endotoxin- (and other bacterial toxins or virulence factors) mediated increase in the release of oxygen intermediates in renal tubular cells. The latter may then be additive to the membrane damage produced in the same cells by the aminoglycosides themselves (Joly *et al.*, 1991).

G. Fever:

Fever *per se* increases renal metabolic demands. When associated with shock, ischemia and the development of foci of tissue necrosis, this enhances aminoglycoside nephrotoxicity by accelerating the course and severity of the toxic insult (Zager, 1988; Spiegel *et al.*, 1990).

2.3.2. **Drug-related-factors**

A. Duration of the aminoglycosides therapy:

A large array of clinical data supports the notion that duration of therapy is a critical factor for developing the clinical manifestation of aminoglycoside-induced nephrotoxicity. The mechanism is probably that kidney (i) does not use all of its nephrons at each time, leaving a subpopulation unaltered which can then be recruited once the main population becomes less functional, and (ii) that kidney proximal tubules are capable of regeneration. If regeneration is insufficient, renal function will be affected once a sufficiently large proportion of all available nephrons have been recruited and intoxicated, which typically may take about 4 to 7 days.

B. Drug choice:

Much dispute has been heard in this area. In the rat model, tobramycin was clearly found to be less toxic than gentamicin both in animals and man (Gilbert

et al., 1978). The respective positions of amikacin and netilmicin (to limit the discussion to the most commonly used aminoglycosides) have been subject to many more controversies. Commercial interests have fueled these largely, but genuine differences in scientific and practical approaches have also been important. Thus, high dose studies, which are necessary in rats to cause overt renal dysfunction, concluded that netilmicin was considerably safer than gentamicin. Likewise, amikacin was also declared much less toxic. The problem may relate to the dosages and to the criteria used. Indeed, when low, clinically relevant doses are used, netilmicin appears as toxic as gentamicin, whereas amikacin stands as a mild or non-toxic drug (Smith *et al.*, 1977; Lerner *et al.*, 1983). The question, which has not been really answered so far, is whether these differences are sufficient to translate into differences of clinical toxicity, in view of the abundance of risk factors discussed above that may make any comparison quite hazardous. As an example, one clinical study found netilmicin less toxic than tobramycin, but the level of toxicity of tobramycin it-self in that study was much lower than was usually reported suggesting that risks factors had been minimized in the studied population. With respect to amikacin, many studies used this antibiotic as a second line drug after failure with another aminoglycoside. Amikacin, indeed, is active against many strains resistant to gentamicin or tobramycin. In case of resistance to the former drugs, change to amikacin will often have taken place after day 3-4 exposure to the first drug. Amikacin-induced toxicity may have been largely contributed by the first treatment and by the greater length of these successive treatments. In studies where amikacin was used as first line agent, toxicity was usually very mild.

c. Frequent dosing intervals:

Numerous clinical trials, the results of which have been bundled in an impressive series of meta-analyses, have shown that a once-daily dosing of aminoglycosides is as at least a safe and as efficacious as the previously recommended method of dividing the daily dose in 2 to 3 administrations at 12

or 8 h interval (Gilbert, 1997). This mode of treatment, which will be discussed in details later in this chapter, does not prevent drug toxicity but may reduce the risk.

D. Concomitant administration of drugs:

The toxicity of aminoglycosides can be enhanced by the co-administration of other drugs and, conversely, other nephrotoxic drugs can amplify the nephrotoxic potential of aminoglycosides. This has been clearly demonstrated for the combinations of aminoglycosides and the glycopeptide antibiotic vancomycin (Wood *et al.*, 1986; Kibbler *et al.*, 1989) or, to a lesser extent teicoplanin (Wilson, 1998), the antifungal amphotericin B (Kibbler *et al.*, 1989; Gilbert D.N., 2000), the anesthetic methoxyflurane (Barr *et al.*, 1973), the immunsupressant cyclosporin (Whiting *et al.*, 1982), and the anticancer drug cisplatin (Salem *et al.*, 1982; Jongejan *et al.*, 1989).

2.4. Handling of aminoglycosides by the kidney

The first step to understand the physiopathology of aminoglycosides nephrotoxicity was made in the seventies by the demonstration of accumulation of aminoglycosides in the renal cortex (Luft and Kleit, 1974; Fabre *et al.*, 1976). This finding was first documented in animals, but later repeatedly confirmed in the human kidney (Edwards *et al.*, 1976; De Broe *et al.*, 1984; Verpooten *et al.*, 1989; De Broe *et al.*, 1991). Autoradiographic, micropuncture and immunocytochemical studies have shown aminoglycosides are primarily taken up and concentrated by S1/S2 proximal tubule cells (Silverblatt and Kuehn, 1979; Wedeen *et al.*, 1983; Molitoris *et al.*, 1993; Pastoriza-Munoz *et al.*, 1984). The amount of aminoglycoside taken up by the renal cortex is only a small fraction of the total administered dose (about 2-5%) (Fabre *et al.*, 1976), but it must be emphasized that aminoglycosides are not metabolized by mammalian cells. All drugs that is retained by the kidney therefore remains chemically unmodified.

2.4.1. **Mechanism of cortical uptake:**

Much attention had been focused on the identification of pathway(s) responsible for uptake of aminoglycosides and the mechanism proposed remained for a long time controversial. The drug can be taken up into the cell from both the luminal and basolateral membrane, although binding and uptake by brush border membrane predominate (Bennett, 1989). Nowadays, the consensus is that megalin, an endocytic receptor expressed on the apical surface of the proximal tubular epithelium, represents the major route of entry of aminoglycosides through the brush border of proximal tubular cells. This has been elegantly demonstrated with mice having genetic or functional megalin deficiency and which do not accumulate aminoglycosides in their proximal tubular cells and are protected against aminoglycoside-induced nephrotoxicity (Schmitz *et al.*, 2002).

2.4.2. **Intracellular handling :**

Following internalization, aminoglycosides traffic via the endocytic system and accumulate primarily in lysosomes. Inside the lysosomes, the aminoglycosides accumulate in very large amount (reaching concentrations that exceed 10-100 time the serum concentration). Recent information obtained both in cell cultures (LLC-PK1 cells) and in vivo (rat kidney) indicates also that a small but quantifiable amount of the internalized gentamicin (5-10%) traffics directly and rapidly from the surface membrane to the Golgi apparatus in both (Molitoris, 1997; Sandoval *et al.*, 1998; Sandoval *et al.*, 2000; Sundin *et al.*, 2001). This new finding for gentamicin is consistent with the movement along the endocytic pathway. It is also consistent with the previously known movement of the shiga or the ricin toxins from the surface membrane to the Golgi apparatus (Sandvig and van Deurs, 1996; Lord and Roberts, 1998; Johannes and Goud, 1998). Recently, it has been shown this small proportion of gentamicin can also accesses the endoplasmic reticulum through a retrograde transport to be further released insides the cytosol where it has been shown associated with the

mitochondria and nucleus (Sandoval and Molitoris, 2004). However, because aminglycosides are polar, most of the drug taken up into proximal tubular cells (whether in lysosomes or in the Gogi apparatus) will stay for considerable time. Half-life of the drug in this tissue which amounts to several days (Fabre *et al.*, 1976) as opposed to the short serum half-life (2 to 3 h) is thus mainly reflecting cell turn-over with a small contribution of true exocytosis.

2.4.3. **Mechanisms of toxicity**

A major difficulty and a point of many controversies has been and still is to ascertain which changes, among the numerous described today, are truly responsible for toxicity. Several hypotheses have been suggested. An intriguing aspect of aminoglycoside nephrotoxicity that must be taken into account, is that very large amount of drugs (usually 10 times the therapeutic doses) are needed in animals in order to cause clear-cut acute tubular necrosis and concomitant alteration of the renal function (Parker R.A., 1982; Gilbert D.N., 2000). This is in sharp contrast with the clinical situation in which a sizeable fraction of patients experience a loss in renal function upon treatment with clinically-acceptable doses (Smith *et al.*, 1980). The question is therefore to establish which subclinical change seen in animal is responsible for toxicity, and how it causes further development of clinical toxicity (Tulkens, 1986). Yet, the demonstration of a sequence of events from a subclinical alteration to overt toxicity is not necessarily a proof of cause to effect relationship. It is indeed possible that only the more drastic changes, such as an frank decrease in glomerular filtration (Baylis *et al.*, 1977), or extended necrosis (Parker R.A., 1982), both seen in animals only at high doses, really cause toxicity. Having this caveat in mind, we will review here the various hypotheses that have received strong experimental support over the last 20 years.

A. Lysosomal alterations.

When in lysosomes, aminoglycosides induce a marked phospholipidosis that has been demonstrated in cell culture models (Aubert-Tulkens *et al.*, 1979),

experimental animals (Knauss *et al.*, 1983; Giuliano *et al.*, 1986; Feldman *et al.*, 1982; Josepovitz *et al.*, 1985) and man (De Broe *et al.*, 1984). This phospholipidosis develops rapidly and involves all major phospholipids, with, however, a predominant increase in phosphatidylinositol on a relative basis (Feldman *et al.*, 1982; Knauss *et al.*, 1983). Accumulation of phospholipids within lysosomes is responsible for the formation of the so-called « myeloid bodies » that were described and linked to toxicity as early as in the mid and late 70's (Kosek *et al.*, 1974; Watanabe, 1978). Phospholipidosis induced by aminoglycosides has been proposed to primarily result from an impaired degradation due to the inhibition of the lysosomal phospholipases A and C and sphingomyelinase (Aubert-Tulkens *et al.*, 1979; Laurent *et al.*, 1990) and to be related to the binding of the cationic aminoglycosides to phospholipids at the acid pH prevailing in lysosomes (Mingeot-Leclercq *et al.*, 1988; Mingeot-Leclercq *et al.*, 1989). The link between lysosomal phospholipidosis and further cell damage has, however, remained largely indirect so far (Laurent *et al.*, 1990). Moreover, some aminoglycosides, such as netilmicin, induce a conspicuous phospholipidosis (Toubeau *et al.*, 1986) without marked necrosis and renal toxicity (Luft *et al.*, 1976). This may, however, be related to differences in dose-effect relationships between netilmicin and other aminoglycosides (Hottendorf *et al.*, 1981).

B. Mitochondrial alterations.

Besides metabolic alterations in lysosomes, aminoglycosides also induce changes in mitochondria, namely a competitive interaction with Mg^{2+} resulting in reduced mitochondrial respiration (Simmons, Jr. *et al.*, 1980; Weinberg and Humes, 1980; Bendirdjian J.Gillaster J. and Foucher B, 1982; Bendirdjian J. *et al.*, 1982) which has been considered as a cause of toxicity. For long, this hypothesis has stumbled on the lack of evidence that aminoglycoisdes could ever reach mitochondria before cell necrosis and *post-mortem* redistribution of the drug stored in lysosomes. Yet, release of oxygen radicals species triggered by aminoglycosides at the level of mitochondria has been proposed as a

potentially important mechanism (Walker and Shah, 1988; Ueda *et al.*, 1993), and traffic of part of the cellular gentamicin to mitochondria in vivo has, now, been evidenced (Sundin *et al.*, 2001). This observation may have a particular significance since polyamines are known to be able to activate mitochondria and cause the release of cytochrome c, an important step leading to apoptosis (Mather and Rottenberg, 2001), a mechanism of cell death that will be discussed hereunder. Proteomic analysis following gentamicin administration has also indicated an energy production impairment and a mitochondrial dysfunction occurring in parallel to the onset of nephrotoxicty (Charlwood J. *et al.*, 2002).

c. Inhibition of protein synthesis:

Aminoglycosides act as antibiotics by inhibiting prokaryotic protein synthesis through binding to ribosomes, blocking peptide synthesis initiation and causing mistranslation (Davies *et al.*, 1965; Tai and Davis, 1979; Carter *et al.*, 2000). Studies indicate gentamicin administered *in vivo* reduces renal cortical endoplasmic reticulum protein synthesis *ex vivo* very rapidly (Buss and Piatt, 1985; Bennett *et al.*, 1988). More recent data point to a major in vivo inhibitory effect of gentamicin on protein synthesis after only 2 days antibiotic administration (Sundin *et al.*, 2001). The mechanism by which this occurs is unknown, but could involve inhibition of nuclear transcription or alteration of endoplasmic reticulum or Golgi-mediated post-translational modifications. An intriguing hypothesis raised by the study of the influence of gentamicin on the expression of specific proteins has also been that failure to translate high levels of mRNA into proportionally high levels of protein would attenuate the expression of stress response gene products, and thus diminish the possibility of recovery in gentamicin intoxication (Dominguez *et al.*, 1996). Recently, gentamicin was found to significantly reduce Na(+)/glucose cotransporter (SGLT1)-dependent glucose transport and to down-regulated mRNA and protein levels of the SGLT1 in pig proximal tubular LLC-PK(1) cells (Takamoto *et al.*, 2003).

D. Inhibition of Na^+/K^+ ATPase:

Aminoglycosides inhibit the Na+, K+-ATPase activity in basolateral membranes (Cronin *et al.*, 1982; Williams *et al.*, 1984), and the binding of the drugs to these membranes has been correlated with toxicities (Williams *et al.*, 1987). Inhibition is, however, seen only when aminoglycosides are presented to the cytoplasmic face of the membrane (Williams *et al.*, 1984). It is not known whether this occurs in vivo at therapeutic doses.

E. Apoptosis

The recent observation that aminoglycosides induce apoptosis *in vivo* at therapeutically-relevant doses (El Mouedden *et al.*, 2000a) has shed new lights on the mechanisms of the early stages of nephrotoxicity. Apoptosis, also called Programmed Cell Death, was first described in 1972 (Kerr *et al.*, 1972) from studies of tissue development kinetics and differentiation, but has now been demonstrated to be a key determinant in cell response to many environmental signals leading to cell death. It is characterized by specific features such as cell shrinkage, increased cytoplasmic density, condensation of chromatin and fragmentation of DNA. It can be triggered in the kidney by a very large array of toxic agents (Davis and Ryan, 1998). In the context of aminoglycosides, rats show a clearly detectable apoptotic reaction in proximal tubules after only 4 days of treatment, which become conspicuous after 10 days (see Figure 6 A). This reaction is dose-dependent, and occurs in the absence of necrosis (El Mouedden *et al.*, 2000a). Gentamicin-induced apoptosis can be also demonstrated on cultured renal (LLC-PK1 [see Figure 6 B], MDCK) and non-renal (embryonic fibroblasts) cells (El Mouedden *et al.*, 2000b).

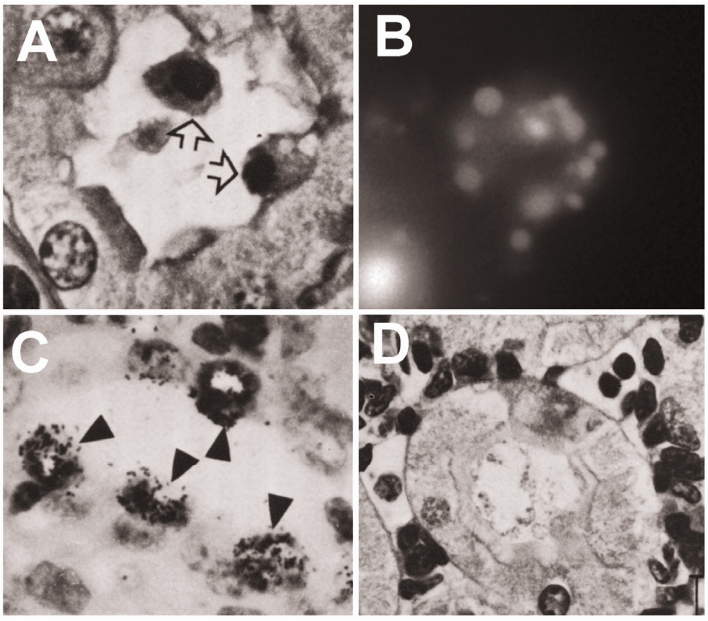


Figure 6: *Morphological changes in rat renal cortex (A,C,D)upon treatment with gentamicin at low doses (10mg/kg, 10 days) and in cultured LLC-PK1 renal cells (B) upon incubation with gentamicin under conditions causing a drug accumulation similar to that observed in rat renal cortex of the animal treated as indicated in A, C and D (approximately 10µg/g tissue) (El Mouedden 2000 229). (A) Typical image of apoptosis (shrinkage necrosis; open arrows) in a seemingly normal proximal tubule; (B) typical image of nuclear fragmentation in a single cell; (C) autoradiographic demonstration of thymidine incorporation in nuclei of proximal tubular cells (arrowheads); (D) peritubular infiltration by endothelial and fibroblasts-like cells. (A, D) hematoxylin-eosin/periodic acid Schifft staining; (B) 4',6'-diamidino-2-phenylindole staining; (C) animals injected with 3H-thymidine one our before sacrifice and tissue sections processed for autoradiographic detection of radioactively-labeled structures followed by light hematoxylin-eosin counterstaining. (A, C, D: reproduced from Laurent 1983 586) with permission; B, unpublished data from H. Servais).*

F. Regeneration

The kidney has a large capacity to regenerate and thereby to compensate for tubular insults. This explains why necrosis and other related processes may go along way without being detected by functional explorations. The importance of this fact is best demonstrated in rats in which fairly high doses (10 times the human doses) can be given for periods as long as more than 40 days. After a first episode of acute renal failure, related to the synchronous necrosis of a large proportion of the proximal tubules, renal function returns to normal as up the animals had become refractive to the toxic effects of the drug (Elliott *et al.*, 1982b; Elliott *et al.*, 1982a). This "resistance" to gentamicin is a state of persistent tubular cell injury obscured functionally by preservation of the

glomerular filtration rate and histologically by asynchrony of cell necrosis and regeneration (Houghton *et al.*, 1986). Tubular regeneration has been extensively studied by means of ^{3}H-thymidine incorporation and other methods, and was shown to occur very early on during treatment even at low, clinically significant doses [see Figure 6 C] (Laurent *et al.*, 1983; Toubeau *et al.*, 1986; Laurent *et al.*, 1988). It may therefore be speculated that all patients exposed to aminoglycosides experience focal losses of tubular tissue (through apoptosis or necrosis), and that the quality and extent of regeneration is what maintains an active renal function or not during treatment. After about 10 days of exposure to gentamicin in subtoxic doses, however, the kidney will already show signs of mild peritubular inflammation and fibroblast proliferation (see Figure 6 D). This will eventually lead to chronic tubulointerstitial nephritis with progressive renal failure. Cessation of treatment is associated with microcystic and inflammatory changes, suggesting that the renal response to tubular injury can be dissociated from the amount of toxin in the renal cortex (Elliott *et al.*, 1982a; Houghton *et al.*, 1988). Both regeneration and assessment of fibrosis and other signs of tubulointerstitial inflammation has therefore be used as surrogate markers in many studies evaluating and comparing aminoglycosides at low doses ((Tulkens, 1986; Hottendorf and Gordon, 1980). The whole process of regeneration / fibrosis induced by aminoglycosides has also been examined within the context of the release of growth factors (Morin *et al.*, 1992; Leonard *et al.*, 1994). While studies aiming at stimulating regeneration have been disappointing, it has now been recognized that macrophages, myofibroblasts, TGF-beta, endothelin and angilotensin II may contribute to the development of renal fibrosis in gentamicin-treated rats (Geleilete *et al.*, 2002).

2.5. Means of protection:

Reducing or protecting against aminoglycosides nephrotoxicity has attracted much effort and attention over the last decade, based either on purely clinical approaches, or on various experimental methods. The former will be discussed

in details because they are the only ones so far that have really allowed decreasing aminoglycoside toxicity in patients.

2.5.1. Clinical approaches:

A. The once-daily schedule:

Up to the late 80's, aminoglycosides were commonly recommended for administration in divided doses over the 24 h period, and typically in q8h or q12 h schedule (i.e. the total daily dose divided in 2 or 3 administrations at 8 or 12 h interval). Kinetic and toxicodynamic studies revealed, however, that this mode of administration resulted in an enhanced drug uptake and toxicity as compared to a once-a-day schedule (Bennett *et al.*, 1979; Giuliano *et al.*, 1986). In parallel, pharmacodynamic studies examining the antibacterial activity of aminoglycosides in vitro (Blaser *et al.*, 1985), in experimental infections (Gerber *et al.*, 1989; Leggett *et al.*, 1990), and in patients (Moore *et al.*, 1987) indicated that a large serum peak concentration to MIC ratio was a key determinant in their efficacy. Aminoglycosides are indeed typically concentration-dependant antibiotics and have a large post-antibiotic effect (Vogelman and Craig, 1986). This led a series of investigators to test the once-a-day schedule first in limited clinical situations from the mid and late 80's (Powell *et al.*, 1983; ter Braak *et al.*, 1990; Tulkens, 1991) to a more wide usage in the mid 90's (Prins *et al.*, 1993; Nicolau *et al.*, 1995) and eventually leading to more than 300 publications to date, and several meta-analyses (Blaser and Konig, 1995; Ferriols-Lisart and Alos-Alminana, 1996; Munckhof *et al.*, 1996). This schedule is now recommended in most cases for reasons of both potentially-improved efficacy and potentally-decreased toxicity (Gilbert, 1997; Gilbert D.N., 2000). As far as toxicity is concerned, the bottom line of all these efforts is nephrotoxicity is usually delayed, although not suppressed with the once-a-day schedule. This is particularly well illustrated by the results presented in Figure 7, where one sees that both the multiple-daily and the once-daily dosing of netilmicin will eventually lead to a similar proportion of patients experiencing nephrotoxic reaction.

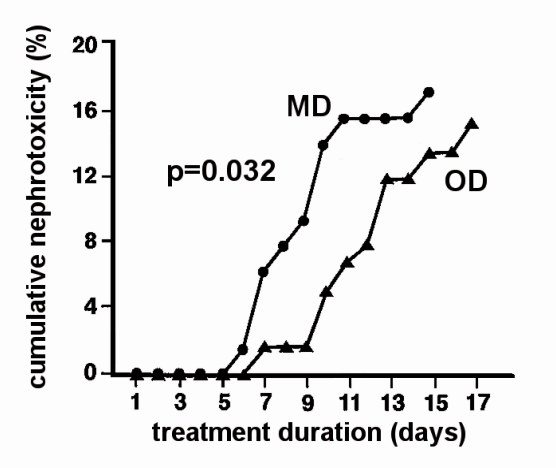


***Figure 7**: Appearance of nephrotoxic reaction in patients given netilmicin once-daily (OD) or on 12 hours or 8 hours multiple doses (MD) (ter Braak 1990 58). Nephrotoxicity was defined as an increase in serum creatinine concentration of more than 50% over baseline. Patients (141) were predominantly elderly subjects with severe bacterial infection and received simultaneously 2g ceftriaxone/day. Netilmicin treatment did not differ significantly in mean daily dose per kg body weight (average 6.6 mg/day) nor duration of therapy between the two treatment arms exept for schedule. Compared with patients receiving conventional doses, patients treated with once-a-day dose had higher serum peak netilmicin levels and lower through levels (Reproduced with minor modifications with permission).*

Patients receiving the drug once-a-day, however, will be safer for 2 to 3 days more than those treated with the conventional schedule. This is understandable since the once-daily schedule will not abolish but only reduce drug uptake by the kidney. Therefore, treating patients for an extended period of time will eventually cause the cortical drug level to reach in both cases the critical treshold that results in tubular insult and ensuing functional damage. This is the basis for the current recommendations to limit aminoglycoside treatments to a maximum of 7 days unless there is clear and defined medical reason to the contrary. It is indeed important to note that pharmacodynamic considerations predict that the once-a-day schedule will be at least as effective, and even perhaps more effective than the divided dose schedule (Blaser *et al.*, 1985; Blaser *et al.*, 1987; Amsden *et al.*, 2000), so that a short course will be effective. When all studies are considered at a fixed time point, the once-a-day schedule appears almost never more, and often less nephrotoxic than the divided-dose schedules [with a global risk factor calculated for 8 studies and a total of 802 patients of 0.9 with a confidence interval of 0.63-1.31 (Verpooten *et*

al., 2003), while being as or more efficacious (Blaser and Konig, 1995) and offering obvious practical advantages, including less necessity of monitoring serum levels (see hereunder). A survey made in the late 90's among 500 acute care hospitals in the US revealed that 74.7 % use the once-daily schedule or a close variant of it (Chuck *et al.*, 2000).

B. Individualized Pharmacokinetic Monitoring:

Individualized pharmacokinetic monitoring represents another approach that has been largely used by clinicians and clinical pharmacists in attempt to optimize aminoglycoside therapy as from the early uses of these drugs to minimize toxicity (Dahlgren *et al.*, 1975; Pancorbo *et al.*, 1982), while ensuring sufficient serum levels to obtain maximal therapeutic effect (Zaske *et al.*, 1982). This has led to intense efforts at designing the most appropriate models and approaches to define "optimal" peak and trough levels that were considered as gold standards and used to compare drug toxicities (Smith *et al.*, 1980). This, however, led sometimes to contradictory results (Pancorbo *et al.*, 1982; Burton *et al.*, 1991; Bertino, Jr. *et al.*, 1993). But it allowed to identify patients at risk (Mullins *et al.*, 1987; Bertino, Jr. *et al.*, 1993), to reassess the value of accepted "normal" therapeutic ranges (McCormack and Jewesson, 1992; Watling and Dasta, 1993), and to minimize costs (Bertino, Jr. *et al.*, 1994). When examined in large patient populations, individualized pharmacokinetic monitoring eventually demonstrated a significant *negative* risk factor of up to 0.42 vs controls, together with substantial savings (Streetman *et al.*, 2001). The outcomes, however, remained blurred by the misconception that a high peak was associated with toxicity, while a minimum trough level was necessary for activity. As we have seen, the converse is true but can only be demonstrated if comparing different schedules of administration.[1]

[1] comparing schedules is essential, since changing the dose but not the schedule modifies simultaneously, and to a similar direction, the C_{max}, the AUC and the C_{min}. These are indeed covariables with respect to the dose, making impossible to ascribe toxicity or efficacy to one of them independently of the others by simply manipulating the dose.

The introduction of the once-daily dosing largely modified the way monitoring was made, by concentrating more on the peak levels (for efficacy), while trough levels being often vanishingly low (at 24 h) tended to be no longer recorded (Cronberg, 1994). Today, optimal true peak levels (i.e. extrapolated at time = 0 h) are set to around 20 mg/L for gentamicin, tobramycin, and netilmicin, and 60 mg/L for amikacin, with trough (24 h) levels lower than 1 and 3 mg/L, respectively, for patients with normal renal function (Gilbert D.N., 2000). These levels will be obtained in most patients, decreasing the necessity of systematic monitoring. However, surveillance may be useful in patients at risk. A nomogram using the 8 h serum value, as an indicator of both the potential peak level (and thereby allowing to adjust the dose) and the elimination constant (to detect impending renal failure) has been developed (Nicolau *et al.*, 1995). It seems fairly popular in the U.S. since about one third of the hospitals using the once-daily schedule have adopted it (Chuck *et al.*, 2000). This nomogram, as others, may, however, not be optimal (Wallace *et al.*, 2002). Application of the principles of individualized pharmacokinetic monitoring to the once-daily schedule (by adjusting the dose interval) may indeed still ensure a further reduction of toxicity while allowing to use larger doses and therefore to potentially increase efficacy (Bartal *et al.*, 2003). Additional reduction of toxicity of the once-daily schedule by selecting the most appropriate time of the day for administration has been reported (Rougier *et al.*, 2003) with early afternoon appearing as optimal. The practicability of this approach needs, however, to be critically assessed.

2.5.2. **Experimental approaches:**

A. Reduction of cortical uptake:

This has been attempted by complexing aminoglycosides with polyanionic substances such as dextran sulfate (Kikuchi *et al.*, 1991) or inositol hexasulfate (Kojima *et al.*, 1990), or anionic β-lactams such as piperacillin (Hayashi *et al.*, 1988) or moxalactam [latamoxef] (Kojima *et al.*, 1990), fosfomycin (Fujita *et al.*, 1983) a, or even simply pyridoxal-5'-phosphate [one coenzyme form of vitamin

B6] (Smetana *et al.*, 1992). Other attempts have been directed at (i) decreasing the electrostatic attraction of aminoglycosides and their brush border binding sites by infusion of bicarbonate (Chiu *et al.*, 1979) to raise urine pH and decrease the polycationic character of aminoglycosides [the pKa of the aminofunctions of the common aminoglycosides span from 5.5 though approx. 8), or (ii) competing with aminoglycosides by means of calcium (Humes *et al.*, 1984) or lysine (Malis *et al.*, 1984). Lack of marked efficacy and/or intrinsic toxicity have, however, prevented all these approaches to be developed in the clinics. Stimulation of exocytosis by fleroxacin (a fluoroquinolone antibiotic) has also been attempted (Beauchamp *et al.*, 1997). Pasufloxacin, an experimental fluoroquinolone, also has shown a protective effect against arbekacin-induced nephrotoxicity, and that this was attributable to a suppression of uptake of arbekacin in cortical renal tubules (Kizawa *et al.*, 2003). The concomitant use of an aminoglycoside and a fluroquinolone to prevent toxicity runs, however, against the rules of the safe and restricted use of antibiotics.

Another approach has been developed more recently by the Industry ReceptIcon (htpp://recepticon.com). They have developed megalin antagonists (the receptor that mediates aminoglycosides uptake) and this molecule is currently under the late preclinical phase for the reduction of aminoglycosides nephrotoxicity. However, this approach needs certainly to be completed to ascertain its safety since megalin mediates the reabsorption of essential components such as vitamins, apolipoproteins or hormones.

B. Prevention of phospholipidosis:

Polyaspartic acid has been shown to protect against both morphological and functional signs of aminoglycoside-induced nephrotoxicty in the rat (Gilbert *et al.*, 1989; Beauchamp *et al.*, 1990a). Further studies showed that this polyanionic peptide enters cells by endocytosis and reaches lysosomes where it forms ion-pair complexes with aminoglycosides, thereby preventing them to interact with phospholipids (Kishore *et al.*, 1990). Interestingly enough, this

results in an increased cortical accumulation of gentamicin but does not alter the pharmacokinetic parameters relevant to the therapeutic effect of aminoglycosides (Whittem *et al.*, 1996). Although potentially promising, the clinical development of polyaspartic acid has been barred by proprietary considerations and by lack of initiatives from the patent holder. Daptomycin (a lipopeptide antibiotic active against Gram-positive organisms) also protects against lysosomal phospholipidosis (Beauchamp *et al.*, 1990b) and neprotoxicity (Wood *et al.*, 1989) in the experimental animal. As polyaspartic acid, daptomycin binds to phospholipid bialayers. However, the contribution of daptomycin to the membrane charge density and its effect on the lipid packing both combine to counteract the inhibition of phospholipase activity due to aminoglycosides (Gurnani *et al.*, 1995), and result in an activation of phospholipases when aminoglycosides are present (Carrier *et al.*, 1998). Daptomycin has been recently approved in the U.S. for clinical usage, evaluations of its potential to reduce aminoglycoside toxicity in patients at therapeutic doses is therefore awaited with interest.

C. Reduction of cell death and increase of cell repair:

This has been attempted with a number of compounds such as desferroxamine (Ben Ismail *et al.*, 1994), methimazole (Elfarra *et al.*, 1994), vitamine E + selenium (Ademuyiwa *et al.*, 1990), lipoic acid (Sandhya *et al.*, 1995), on the one hand, and ulinastatin (Nakakuki *et al.*, 1996) , fibroblast growth factor 2 (Leonard *et al.*, 1994) and the heparin-binding epidermal growth factor (Sakai *et al.*, 1997). No clinical data, however, is available.

D. Protection against vascular and glomerular insults:

This can be obtained with calcium channel blockers (Lortholary *et al.*, 1993), but the intrinsic pharmacological properties of these agents has prevented any clinical development. Trapidil, an antiplatelet and vasodilator agent may also protect by antagonism of platelet-derived growth factor (PDGF), vasodilatation,

inhibition of trombocyte aggregation, and/or NO release (Buyukafsar *et al.*, 2001).

2.6. Concluding remarks

Although having attracted so much attention from laboratory and clinical researchers, the basic mechanims of aminoglycoside nephrotoxicity, and especially the biochemical events leading to cell damage and glomerular dysfunction still remain poorly understood. However, the observation that these antibiotics are able to induce cell death by apoptosis *in vitro* but also *in vitro* at doses relevant to clinical situations have allowed new approaches to explain their nephrotoxicity. As mentioned earlier, apoptosis is a type of cell death, which is executed by specialized enzymes, the caspases, but also highly regulated by the proteins of the Bcl-2 family. Apoptosis can be initiated by different molecular pathways, which have been initially divided into two major ones: the receptor-mediated (or extrinsic) pathway and the organelle-mediated (or intrinsic) pathway. The importance of this process in gentamicin nephrotoxicity is further supported by the observation that numerous nephrotoxins can induce this type of cell death (Ueda *et al.*, 2000) but also by the observation that tubular cell death by apoptosis is probably an important contributor to the development of acute renal failure induced by these nephrotoxins (Ortiz *et al.*, 2003; Ueda *et al.*, 2000).

3. CELL DEATH: APOPTOSIS

As **apoptosis** seemed to be **a key contributor of aminoglycosides nephrotoxicty**, here is an insight of this particulate phenomenon in order to further understand how these agents, and in particular gentamicin, is able to induce such process.

The cell death exposed to a toxic substance has been shown to result from two major processes: apoptosis and necrosis. The pathway that is followed by the cell is dependent on both the nature and severity of the insults, evolving from apoptotic to necrotic form of cell death. Thus its is now recognized that the same insult in a mild form can lead to apoptosis and when severe can lead to necrosis (Healy *et al.*, 1998; Ueda *et al.*, 2000).

Apoptosis is described as an active and a genetically regulated form of cell death, it is an evolutionary conserved physiological process used by an organism to selectively eliminate cells that are no longer needed such as damaged or infected cells (Wyllie *et al.*, 1980). The term apoptosis was used in Greek to denote a “falling of” as leaves from a tree (Kerr *et al.*, 1972). This term indeed connotes a controlled physiologic process of removing individual components of an organism without destruction or damage to the organism. Apoptosis is characterized by well-defined morphologic characteristics: chromatin condensation in one or more masses in the nucleus, this event starts along the nuclear membrane and forms a crescent or ring-like structure (chromatin margination); this condensation is followed by the shrinkage and the fragmentation of the nucleus into oligonucleosome-sized DNA fragments by specialized nucleases (Wyllie *et al.*, 1980). Other characteristics are cytoplasmic condensation and finally cellular fragmentation into membrane-bound fragments. These fragments are called apoptotic bodies and are taken up by other cells and degraded in phagosomes (Kerr *et al.*, 1972). Apoptosis plays a crucial role during embryogenesis but also later in the maintenance of homeostasis, the clearance of old cells, the maturation and function of the

immune system (Lockshin and Zakeri, 2001; Danial and Korsmeyer, 2004; Krammer, 2000).

Necrosis has been characterized as passive, accidental cell death characterized by a cell swelling with early loss of plasma membrane integrity, major changes in the organelles, swelling of the nucleus with flocculation of the chromatin. This death results from environmental perturbations with uncontrolled release of cellular components spilling into the surrounding tissue space evoking an inflammatory response (Kerr *et al.*, 1972).

Numerous specific changes associated with apoptosis result from the activation of particular enzymes, so-called **caspases**, which cleaves vital proteins in cell but also activate more specific enzymes such as caspases-activated DNAse (CAD) which is the major enzyme responsible for DNA fragmentation. If these enzymes are key components of the apoptotic signalling, the **proteins of the Bcl-2 family** are also critical contributor by regulating the apoptotic process. Among the Bcl-2 family proteins, some of them are anti-apoptotic such as Bcl-2 and Bcl-xl while others possess pro-apoptotic functions such as Bax and Bak.

3.1. Key component of the apoptotic signalling

3.1.1. Caspases

Destruction of cells during apoptosis requires the activation of caspases, which belong to a family of Aspartate-specific Cystein proteases (Alnemri *et al.*, 1996). Caspases are synthetized as a single-chain of inactive zymogens, consisting of four domains, a NH_2 pro-domain of variable length, a large subunit of about 20kDa, a small subunit of about 10 kDa and a linker region connecting these catalytic subunits. Proteolytic cleavage of the caspase precursors results in the separation of large and small subunits with the formation of a heterotetrameric complex (the active enzyme) consisting in two small and two large subunits (Wolf and Green, 1999). Caspases differ in the length and sequence of their NH_2-terminal prodomain which is either short (20-30 amino acids) or long (more than 90 amino acids).

Fourteen caspases have been identified so far, eleven in human (see figure 8) three of which are involved in cytokine activation and inflammation and seven of which have their major role in apoptosis (Riedl and Shi, 2004). Based on their function and their structure, caspases have been divided into four groups.

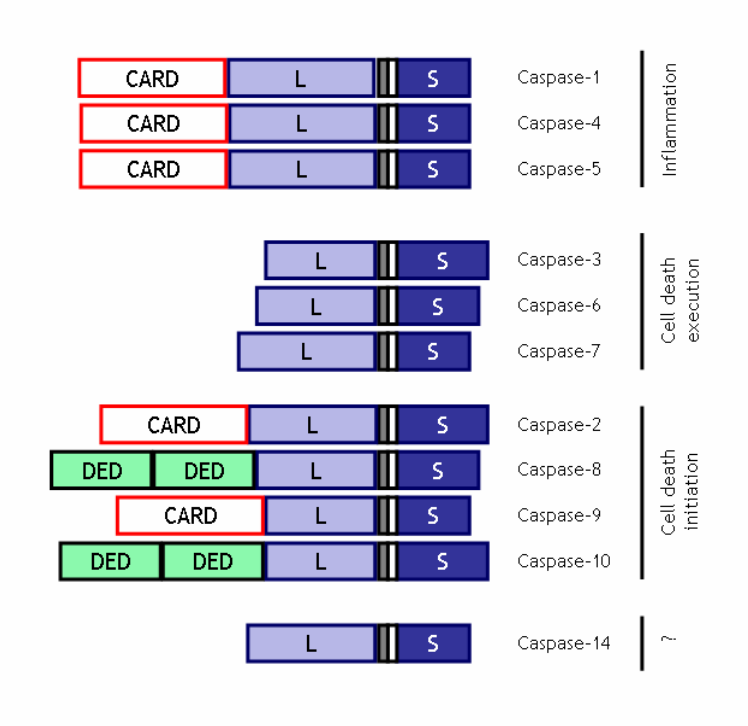


Figure 8: *Schematic representation of human caspase structure. Caspases are cystein proteases that all contain a small (S) and a large (L) subunit including the active site. Grey domain between L and S subunits represents the catalytically active cystein residue. The initiator caspases contain a pro-domain (containing DED or CARD domain) which mediate their interaction with adaptator proteins upon apoptotic stimuli, leading to their activation.*

The initiator caspases have pro-domains that are of two types: the caspase activation recruitment domain (CARD) found in caspase-2, -9 and -12, and the death effector domain (DED) found in caspase-8 and -10. The effector caspases are able to directly degrade multiple substrates including the cellular structural and regulatory proteins leading to characteristic death by apoptosis. Initiator caspases are also cleaved upon activation, similarly to the effector caspases, but this cleavage seemed neither required nor sufficient for their activation (Boatright and Salvesen, 2003). The activation of effector caspases can not only been caused by initiator caspase but also by other non-caspase proteases including cathepsins, calpains and granzyme (Vancompernolle *et al.*,

1998; Stoka *et al.*, 2001)Johnson (Johnson, 2000). Caspase-1 and caspase-4 and -5 have similar structures and are predominantly involved in the maturation of proinflammatory cytokines. At this stage, simplified apoptotic pathway can be summarized as illustrated in figure 9.

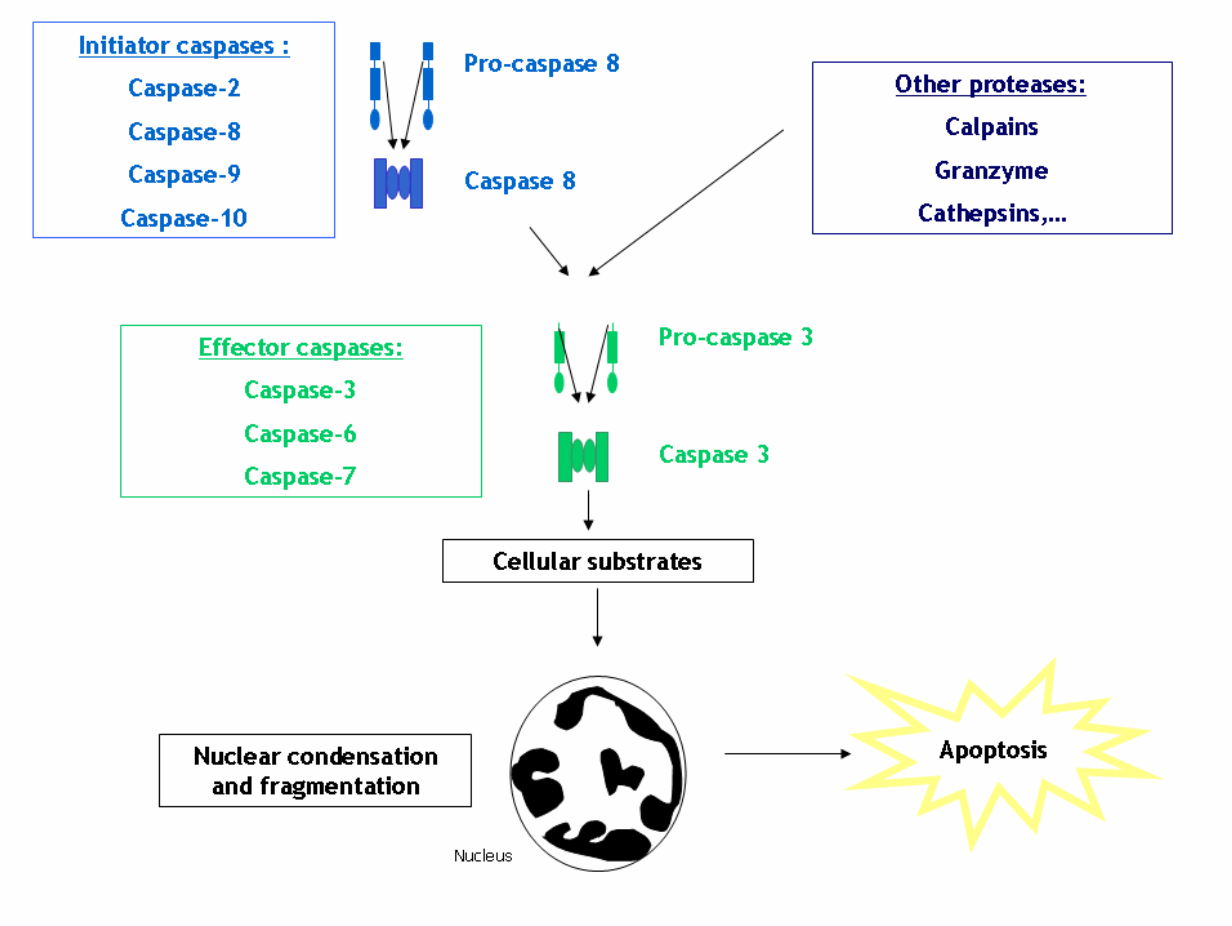


Figure 9: *Schematic representation of caspases activation and apoptosis induction.*

Different types of molecular pathways can lead to activation of these caspases and will be developed later.

3.1.2. Proteins of the Bcl-2 family

Eventhough caspases are the key initiators and executors of apoptosis, the proteins of the Bcl-2 family certainly fulfil a major role in the orchestration of apoptosis. Indeed, they are now recognized as key modulators of apoptosis. Their action site was first thought to be the mitochondria (Gross *et al.*, 1999; Tsujimoto, 2003), however, accumulating evidences have now shown that they

also act in the regulation of the endoplasmic reticulum (ER) pathway (Nutt *et al.*, 2002) and more recently, their involvement in the lysosomal pathway has been established (Kagedal *et al.*, 2005). This family comprises proteins with cell death inhibiting and cell death activating properties. They are characterized by the presence of conserved sequence motifs, named Bcl-2 Homology (BH) domains (see figure 10).

Anti-apoptotic members such as **Bcl-2 and Bcl-xl** share all the four BH domains, BH1 to BH4. Bcl-2 homologues have also been found in some viruses, adenovirus (E1B-19K), Epstein-Barr (BHRF), African swine (LMW5-HL), Herpes virus (ORF16). **Pro-apoptotic proteins** can be divided into two subgroups: the **"multidomain" proteins such as Bax and Bak** which contain BH1, BH2 and BH3 domains and the **"BH3-only" proteins among them Bid, Bim or Bik** which are largely unrelated in sequence to Bcl-2 anti-apoptotic members. BH3 domain is essential for their pro-apoptotic properties.

How exactly Bcl-2 and its anti-apoptotic homologues promote cell survival is not clear (Cory *et al.*, 2003), but mouse genetic studies indicate that the survival of every cell type requires protection by at least one Bcl-2 homologue (Cory *et al.*, 2003; Ranger *et al.*, 2001). The BH1-3 domains of the anti-apoptotic proteins form a hydrophobic groove through which they can bind the BH3 domain of the proapoptotic Bcl-2 family members (Muchmore *et al.*, 1996; Petros *et al.*, 2001). Bcl-2 and homologues may function both by sequestering active BH3-only proteins and by restricting Bax/Bak oligomerization, thereby setting an activation barrier for the induction of apoptosis and limiting inappropriate cell death.

While several Bcl-2 family proteins possess ion channel activity in lipid bilayers, only the multidomain proapoptotic proteins Bax and Bak can renders membranes permeable to cytochrome c and larger molecule (Korsmeyer *et al.*, 2000; Kuwana *et al.*, 2002). These two proteins exhibit a degree of functional redundancy, as ablation of both *bax* and *bak* genes in mouse results in

apoptosis resistance whereas single *bax* or *bak* knock-outs are susceptible to apoptosis induction. Indeed, tissues and cells deficient in both genes are resisitant to most stimuli (Scorrano *et al.*, 2003; Wei *et al.*, 2001; Lindsten *et al.*, 2000; Knudson *et al.*, 1995). These two proteins do not behave identically, however, since in non-apoptotic cells Bax is mostly free in the cytosol while Bak is constitutively localized in membrane of mitochondria and endoplasmic reticulum (Zong *et al.*, 2003)

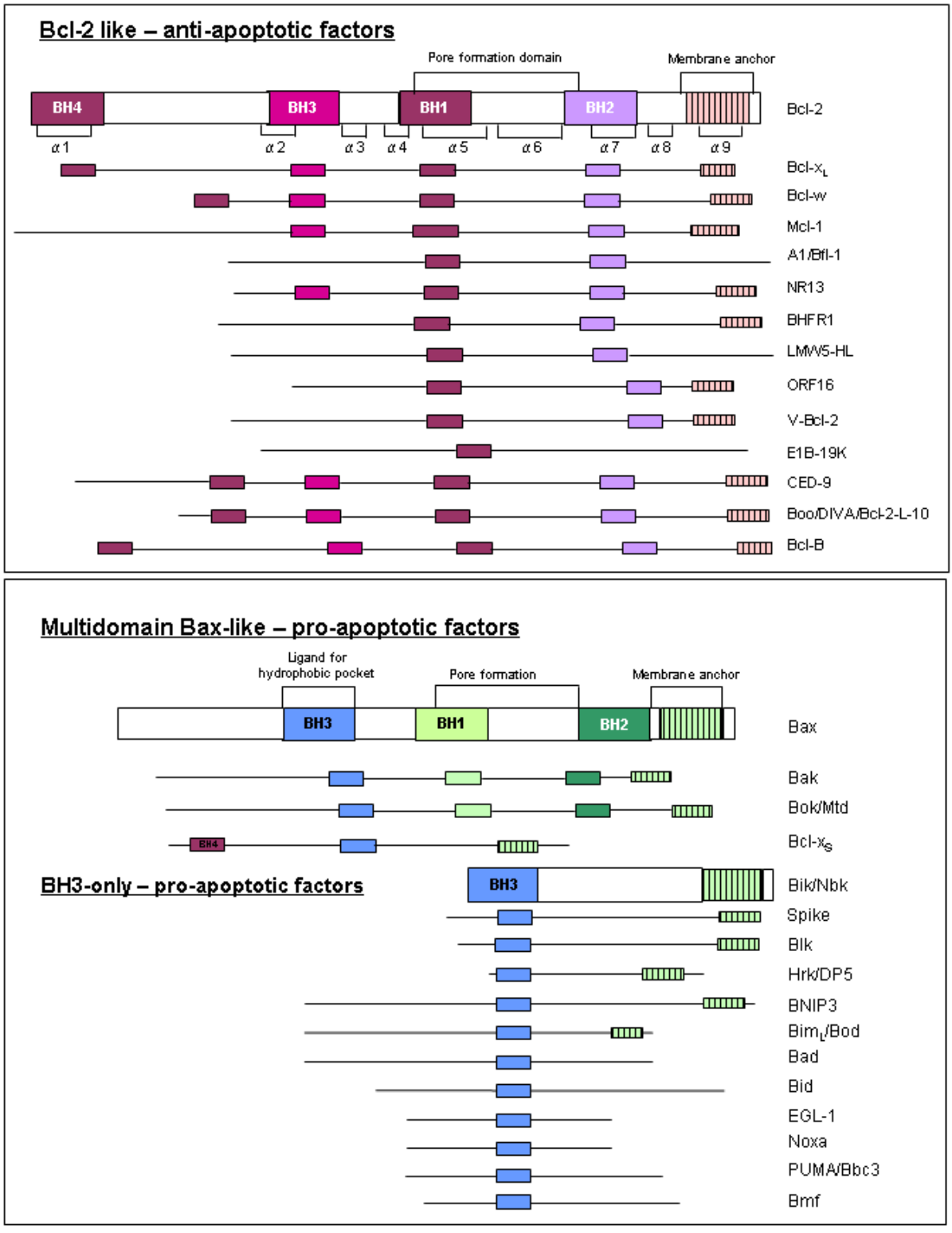


Figure 10: *Classification of the Bcl-2 family. The BH3 domain in the pro-apoptotic members is a ligand for the hydrophobic pocket formed by the BH1-BH3 domains of the survival factors. α-5/α-6 overlap with BH1 domain and are pore forming units based on structural homology with bacterial toxins. The C-terminal membrane anchor is a hydrophobic, single membrane spanning amino acid 17-23 α-helix (α-9) that tail-anchors the proteins in intracellular membrane. Figure 10 is adaptated from Borner(Borner, 2003).*

Bax, which is one of the major pro-apoptotic proteins, was identified by co-immunoprecipitation with Bcl-2 (Oltvai 1993 609). As described before, it belongs to the subgroup of multidomains pro-apoptotic proteins of the Bcl-2 family proteins with BH1, BH2 and BH3 domains. The N-terminus is relatively non-structured, and although a BH4 domain was initially not predicted by amino acid sequence, the relative orientation of the equivalent region in Bax in respect to the rest of the protein is identical to that in Bcl-xl. The structure of Bax consists of nine α-helices (see figure 11).The overall fold of Bax closely resembles that of Bcl-x (Muchmore 1996) with seven amphipathic α helices clustered around two central, mostly hydrophobic α-helices.

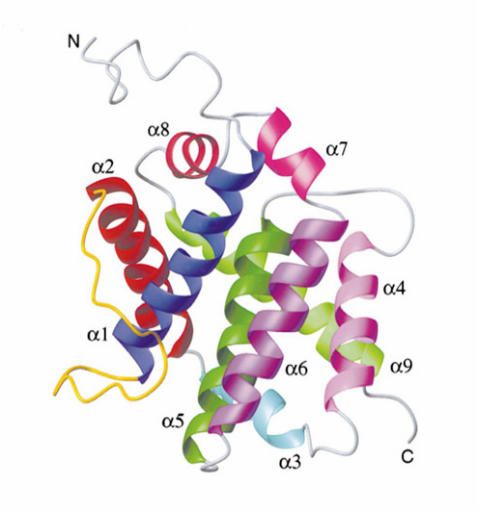


Figure 11: *3-D structure of Bax. From Suzuki (Suzuki et al., 2000)*

An important difference between anti-apoptotic Bcl-xl and pro-apoptotic Bax was found in the BH3 domain. In Bax, this helix is less packed to the hydrophobic core than in Bcl-xl. This makes it easier for the domain to rotate around its axis to expose the residues away from hydrophobic core, making them available for binding to the hydrophobic grooves of Bcl-2 like survival factors (Sattler *et al.*, 1997). This flexibility of the BH3 domain is crucial for the pro-apoptotic activity of Bax because swapping this region from Bax to the anti-apoptotic Bcl-2 converted Bcl-2 to a death agonist despite the presence of a BH4 domain (Hunter and Parslow, 1996).

In unstimulated cells, Bax is a mainly soluble monomeric protein present in the cytosol or loosely associated with mitochondria (Antonsson *et al.*, 2000). In response to apoptotic stimuli, Bax undergoes specific conformational changes which allow its targeting and insertion to the outer membrane of the mitochondria (Cory and Adams, 2002). Moreover, it has been also demonstrated that Bax can be targeted to other intracellular organelles including the ER (Germain and Shore, 2003) and lysosomes (Kagedal *et al.*, 2005). On the mitochondrial level, the insertion of Bax seems to trigger the formation of homo-oligomers allowing the critical release of the proapoptotic factors sequestred in the intermembrane space of mitochondria. Different stimuli have been proposed to induce a conformational change of Bax and/or of its translocation to mitochondria (figure 12): (1) BH3-only proteins Bim (Yamaguchi *et al.*, 2002; Marani *et al.*, 2002), Bid (Desagher *et al.*, 1999; Roucou *et al.*, 2002; Perez and White, 2000), Bad (Tafani *et al.*, 2001), PUMA (Liu *et al.*, 2003) and more recently MAP-1 (Baksh *et al.*, 2005; Tan *et al.*, 2005), (2) some kinases, such as JNK (Yu *et al.*, 2003; Okuno *et al.*, 2004) or p38MAPK (Van Laethem *et al.*, 2004; Ghatan *et al.*, 2000), (3) some proteases such as cathepsin D (Bidere *et al.*, 2003) or the calpains (by cleaving Bax to p18Bax) (Karlsson *et al.*, 2004), (4) a rapid increase of the intracellular pH (Belaud-Rotureau *et al.*, 2000; Khaled *et al.*, 1999) or at the contrary a decrease in cellular pH (Ahmad *et al.*, 2004; Xie *et al.*, 1998), (5) an increase in Bax-cell content (Choi and Singh, 2005; Fernandez *et al.*, 2003; Nam *et al.*, 2001) or the activation of acidic sphingomyelinase (Kashkar *et al.*, 2005) or simply the interaction of Bax and lipids (Yethon *et al.*, 2003).

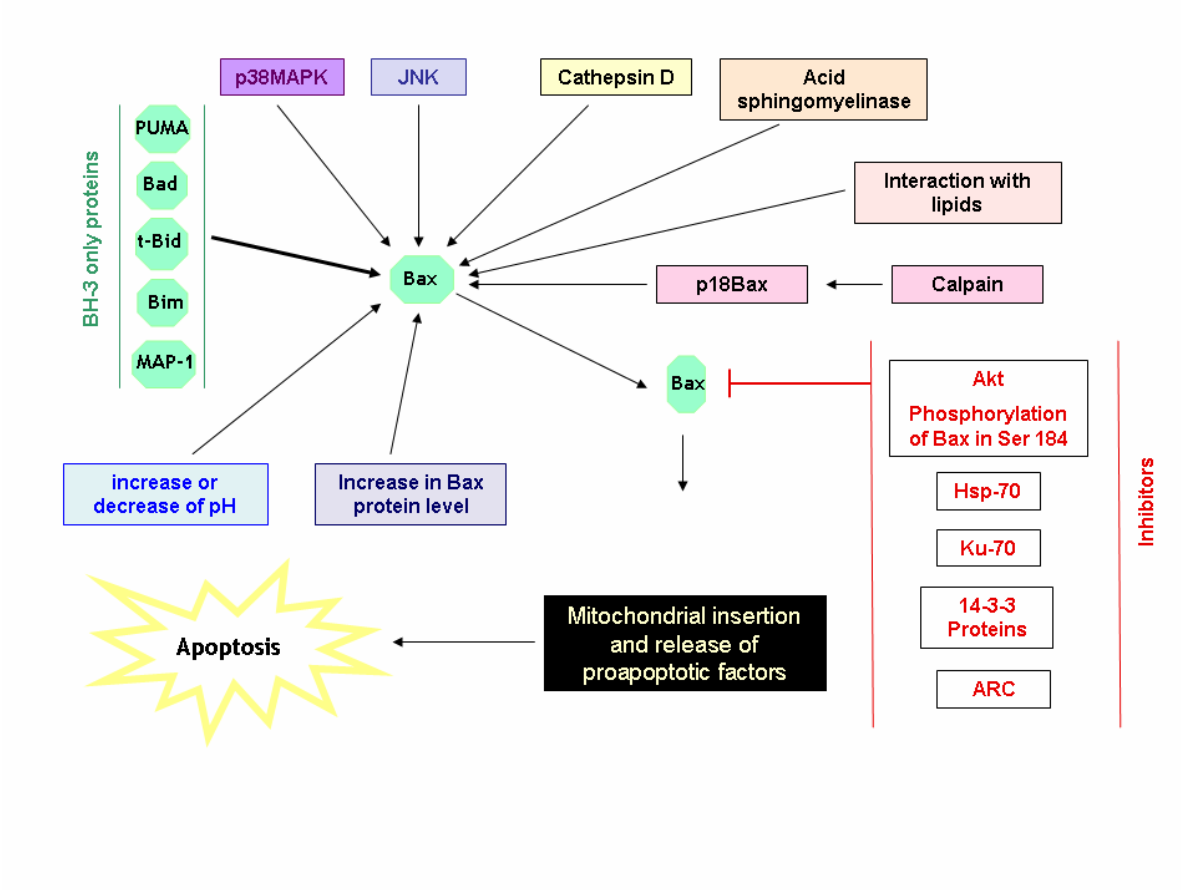


Figure 12: *Cellular events that have been shown to induce directly or indirectly the activation of pro-apoptotic Bax by inducing a conformational change leading to insertion into mitochondrial membranes.*

Bax can be regulated by the Bcl-2-like anti-apoptotic proteins or by interaction with several cytoplasmic inhibitors, including Ku-70 (Sawada *et al.*, 2003), 14-3-3 proteins (Tsuruta *et al.*, 2004), humanin peptide (Guo *et al.*, 2003), and hsp70 (Stankiewicz *et al.*, 2005), ARC (Apoptosis Regulator with a CARD domain) (Gustafsson *et al.*, 2004), or also following phosphorylation by AKt on its Ser184 (Gardai *et al.*, 2004).

3.2. Molecular pathway leading to apoptosis

3.2.1. Receptor-mediated (extrinsic) pathway

Death receptors belong to the TNF receptor gene superfamily which consists of more than 20 proteins with a broad range of biological functions including regulation of cell death and survival, differentiation or immune regulation (Ashkenazi and Dixit, 1999; Walczak and Krammer, 2000; Ashkenazi and Dixit, 1998). Death Receptors are defined by a cytoplasmic domain of about 80 amino acids called “death domain”, which play a crucial role in transmitting death signals from the cell surface to the intracellular signalling pathways. Fas (APO-1/CD95), TRAIL-R1 and TRAIL-R2, TNF Receptor 1 (TNFR1) are the most studied in death signalling pathway. DR3 and DR6 have also been involved in apoptosis signalling cascade (Fulda and Debatin, 2004).

Fas death signal has been widely studied and is as model for the description of this pathway (see figure 13). The binding of Fas Ligand to Fas receptor triggers its oligomerization and induce the recruitment of Fas-Associated Death Domain protein (FADD) and pro-caspase-8 to form a complex named DISC (Death Inducing Signaling Complex) (Kischkel *et al.*, 1995; Boldin *et al.*, 1996; Muzio *et al.*, 1996). Caspase-8 molecules are then activated and reach the cytosol where they can activate the effector caspases. Caspase-8 has also the ability to cleave a BH3-only protein, Bid into truncated-Bid (t-Bid) then allowing its translocation to the mitochondria (Li *et al.*, 1998; Luo *et al.*, 1998).

For Fas signalling pathway, two distinct prototypic cell types have been identified (Scaffidi *et al.*, 1998). **In type I cells**, caspase-8 is activated upon death receptor binding at the DISC in quantities sufficient to directly activate downstream effector caspases such a caspase-3. **In type II cells**, however, the amount of active caspase-8 generated at the DISC is insufficient to activate caspase-3 but sufficient to activate Bid which in turn will activate the mitochondrial pathway.

A similar cell type-dependent organization of the TRAIL signalling has been described (Fulda *et al.*, 2001). The signalling pathway for the TNF-related apoptosis inducing ligand (TRAIL) (TRAIL-R1 and TRAIL-R2 involved in apoptosis) is similar to the signalling pathway for Fas, with the formation of the DISC complex, the recruitment of FADD and caspase-8 and the activation of downstream effector caspases. Beside caspase-8, caspase-10 could also be recruited in the TRAIL DISC but its importance in apoptosis is subjected to controversies (Kischkel *et al.*, 2001; Sprick *et al.*, 2002).

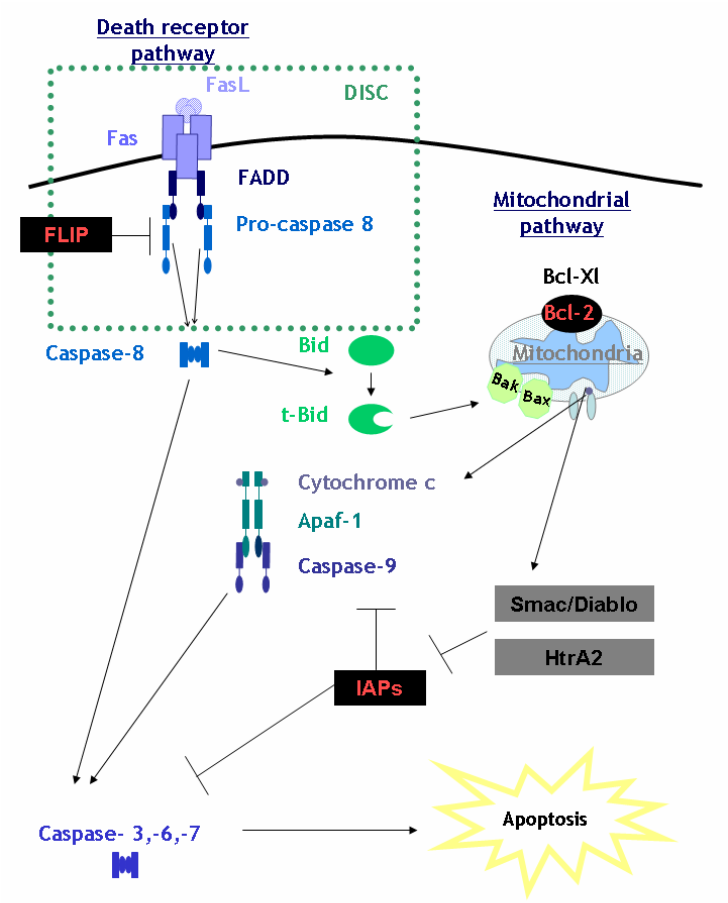


Figure 13: *Receptor-mediated death pathway. The binding of the ligand (FasL) to its death receptor (Fas) recruits the adaptator molecule FADD and pro-caspase-8 to form a specific complex named DISC (Death Inducing Signalling Complex). This allows the activation of pro-caspase-8 by cleavage. Caspase-8 can activate directly the effector caspases such as caspase-3 but this can occur indirectly by activation of the mitochondrial pathway by the cleavage of Bid in truncated-Bid (t-Bid). T-bid triggers the release of cytochrome c and the activation of caspase-9 which in turn activates caspase-3. Smac/Diablo and Omi/HtrA2 are released from the mitochondria and contribute to apoptosis induction by inhibiting the IAPs, the cytosolic endogenous caspase inhibitors.*

For TNFR1, the situation is quite different since the ligation of TNF-α (their ligand) can activate several different signalling pathways depending on the specific adaptator molecule recruited to the activated receptor. Binding of TNFR1 by TNF-α results in receptor trimerization and release of the inhibitory protein SODD from the intracellular domain of TNFR1. This in turn enables the recruitment of the adaptor protein TRADD, which serves as a common platform adaptor for several signalling molecules that mediate the different biological functions of TNF-α. For the transduction of death signals, FADD couples the TNFR1-TRADD complex and recruits caspase-8. TNFR2 has been shown to play an important role in the regulation of apoptosis through TNFR1 [reviewed in (Gupta, 2001)].

Signalling by death receptors can also be negatively regulated by a particular protein, FLIP which has been found in two forms $FLIP_L$ (long form) and $FLIP_S$ (short form). FLIP can be recruited to the DISC instead of procaspase-8, -10 resulting in the inhibition of caspase activation (Ivanov *et al.*, 2001).

3.2.2. **Organelle-mediated (intrinsic) pathway**

A. Mitochondrial pathway

Numerous pro-apoptotic signal transduction and damage pathways converge on mitochondrial membrane to induce their permeabilization. The precise mechanisms by which the permeability of the outer mitochondrial membrane becomes compromised during apoptosis and by which the apoptogenic factors are released have remained controversial, although it is clear that this process is governed by the Bcl-2 family of proteins and in some cases by caspases themselves.

Between all the Soluble Intermembrane Mitochondrial Proteins (SIMPs), cytochrome c was the first molecule shown to be released from the mitochondria during apoptosis. Once in the cytoplasm, cytochrome c binds with

apoptosis protease-activating factor-1 (Apaf-1), dATP and pro-caspase-9 to form the apoptosome (Liu *et al.*, 1996; Li *et al.*, 1997; Zou *et al.*, 1997; Cain *et al.*, 2000). The formation of the apoptosome leads to the activation of caspase-9 and finally to the activation of downstream effector caspases (caspases-3, -6, -7). This enzymatic complex appears to be regulated by XIAP, which has been shown to bind the activated form of caspase-3 and caspase-9, blocking the apoptosome-mediated caspase activation (Bratton *et al.*, 2001). Smac/DIABLO and Omi/HtrA2, two other SIMPs, antagonize cytosolic IAPs (Inhibitor of Apoptosis Protein), whose role is to bind and inhibit cytosolic caspases (Verhagen *et al.*, 2000; Suzuki *et al.*, 2001). Omi/HtrA2 has recently been shown to induce caspase-independent cell death via its serine protease activity by direct association with cell surface death receptor (Blink *et al.*, 2004; Li *et al.*, 2002b). In addition to SIMPs that trigger or enhance caspase activation, proteins such as AIF and endonuclease G are also released from mitochondria and cause caspase-independent DNA fragmentation. Both AIF, endonuclease G and caspase-activated DNase CAD, appear to work in conjunction to complete chromatin condensation and fragmentation, where AIF seemed to be involved in large-scale DNA fragmentation and peripheral chromatin condensation (Susin *et al.*, 1999; Susin *et al.*, 2000).

Even if we know more precisely the identity of released component of the mitochondria during apoptosis, the mechanism by which they are released is subject to controversy and different hypotheses have been raised (see figure 14):

The first mechanism proposed was the **involvement of the PTP (Permeability Transition Pore).** This pore is constituted by the VDAC (voltage-dependent anion channel) and HK (hexokinase), both located in the outer mitochondrial membrane, by the ANT (adenine nucleotide translocase) located in the inner mitochondrial membrane, by the cyclophyllin D located inside the matrix and finally by creatine kinase CK which links both VDAC and ANT proteins. Recent studies in cyclophilin D-deficient animals have shown that this molecule is

important for mitochondrial permeabilization (Baines *et al.*, 2005; Basso *et al.*, 2005). At the opposite, ANT seemed not to be essential for the opening of the PTP (Kokoszka *et al.*, 2004). Opening of this pore is thought to result in mitochondrial swelling and rupture of the outer membrane leading to the non-specific release of the soluble intermembrane mitochondrial proteins (SIMPs) (Kim *et al.*, 2003).

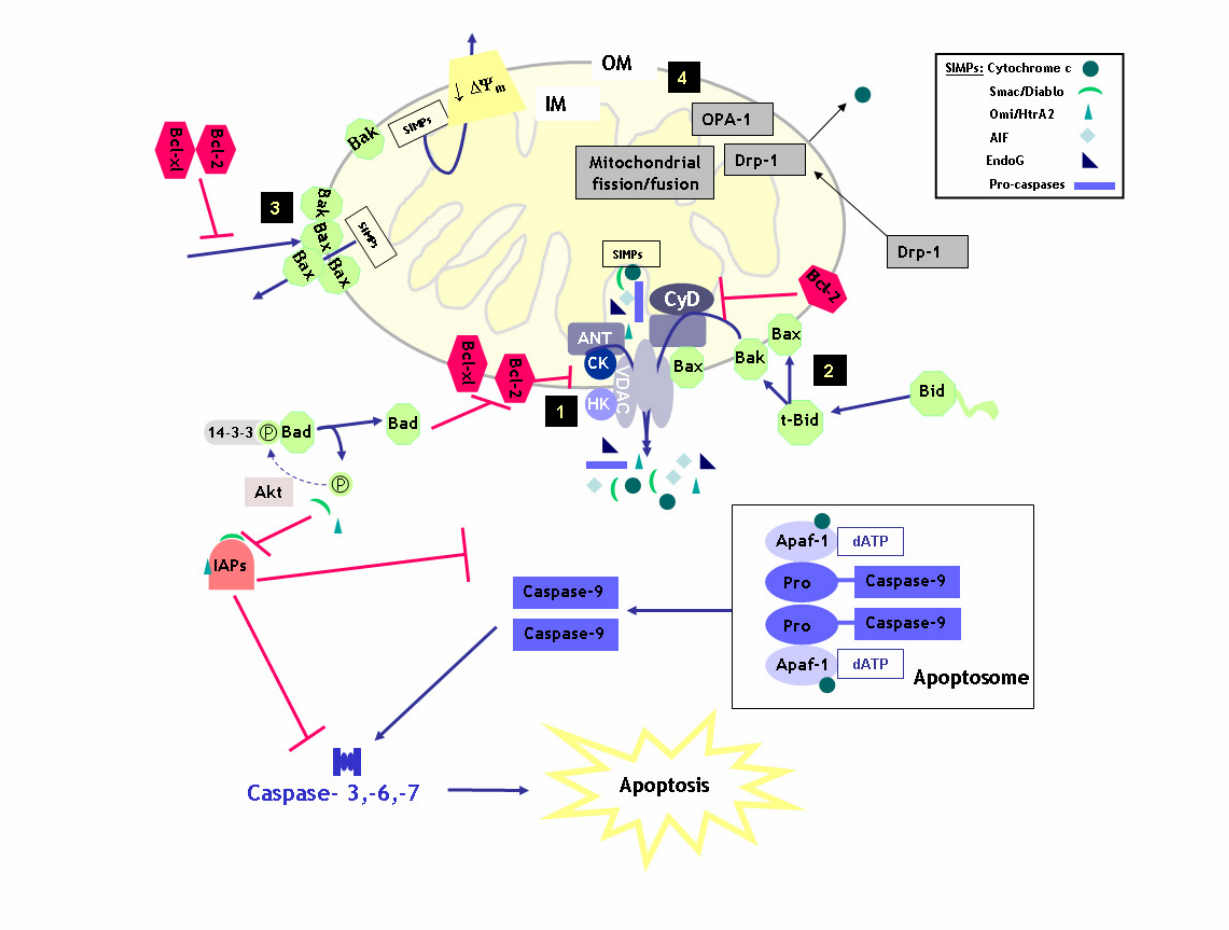


Figure 14: *Mitochondrial activation during apoptosis leads to the release of pro-apoptotic components (also named the SIMPs for Soluble Intermembrane Mitochondrial Proteins) and includes Smac/diablo, cytochrome c , AIF, endonuclease G, pro-caspases and Omi/HtrA2. The release of cytochrome c leads to the formation of a complex including also Apaf-1, pro-caspase-9 and dATP called the apoptosome, the formation of which leads to caspase-9 activation and further caspase-3 activation and cell death. Four hypotheses have been proposed to explain the release of the SIMPs: (1) the release resulted from the opening of specialized pores, the Permeability Transition Pore (PTP) composed by the the Voltage-dependent Anion Channel (VDAC), Adenine nucleotide translocase (ANT) cyclophylin D (CyD), creatine kinase (CK) and hexokinase (HK), (2) the opening of the PTP pores are under strict control of Bcl-2 family proteins, (3) pro-apoptotic Bcl-2 family proteins (such as Bax and Bak) form pores after insertion in mitochondria and (4) the release of SIMPs could also result from a change in the structure and dynamics of mitochondria through fission/fusion mechanisms.*

Secondly, a variation of this model has been proposed upon the demonstration of an **interaction between pro-apoptotic Bcl-2 family member and constituents of the PTP pores** as it has been shown for Bax and ANT (Marzo *et al.*, 1998) but also Bax and VDAC (Shimizu *et al.*, 1999).

A third hypothesis has been proposed and is based on sequence homology with certain bacterial toxins. Indeed, **members of the Bcl-2 family of proteins** such as Bax (Antonsson *et al.*, 1997) and t-Bid (Schendel *et al.*, 1997) seemed to have the ability to **insert into membrane and form channels**. Bax and Bak activation results in their homooligomerization within the outer membrane, with subsequent release of SIMPs and induction of apoptosis (Eskes *et al.*, 2000; Wei *et al.*, 2000). This third hypothesis is also supported by experiments performed with artificial membranes composed of mitochondrial lipids. The channel-forming ability of Bax has been shown to be under the regulation of t-Bid and to induce cytochrome c release from liposomes, an activity dependent on the mitochondrial lipid, cardiolipin (Kuwana *et al.*, 2002).

More recently, **modulators of mitochondrial structure and dynamics** have also been proposed to explain mitochondrial release of pro-apoptotic factors. Between these modulators, Drp-1, a GTPase, seemed to play an important role. Indeed Youle and colleagues found that Drp-1 participate in apoptotic mitochondrial fission (Frank *et al.*, 2001). Specifically, Drp-1 translocate from the cytosol to the mitochondrial outer membrane at the onset of apoptosis. Furthermore, dominant-negative Drp-1 blocks staurosporine-induced mitochondrial fission, loss of mitochondrial potential, cytochrome c release and cell death (Frank *et al.*, 2001). OPA1, another GTPase involved in mitochondrial fusion which localizes in the intermembrane space of mitochondria, has also been shown to be involved in mitochondrial apoptosis (Griparic *et al.*, 2004), and its down-regulation induces mitochondrial fragmentation, cytochrome c release and caspase activation (Olichon *et al.*, 2003). Structure and dynamics of mitochondria could also been under the control of Bcl-2 family proteins and could also be related to the precedently cited hypotheses. To trigger Bax and

Bak homooligomerization in the outer membrane of mitochondria, tBid induces a striking remodelling of mitochondrial structure, associated with induction of membrane curvature and mobilization of the cytochrome c stores in cristae (Scorrano *et al.*, 2002).

B. Lysosomal pathway

Lysosomes are acidic membraned-bound organelles involved in the degradation of extracellular materials internalized by endocytosis and intracellular materials derived from within the cells by autophagy (de Duve, 1983; Hunziker and Geuze, 1996). The destruction of internalized material is executed by the numerous lysosomal hydrolases such as cathepsins. Among the 11 human cathespins described so far (Cathepsin B, H, L, S, F, K, C, W, X, V and O), cathepsin B and L are ubiquitously expressed and are the most abundant in lysosomes together with the aspartic proteases cathepsin D (Turk *et al.*, 2002b). The processing of the proenzymes into the catalytically active form usually occurs within the lysosomes (Ishidoh and Kominami, 2002).

Even though lysosomes have been first described to be involved in necrosis and autophagy, it clearly appears now that they can also mediate death by apoptosis in several models of apoptosis. The key factor in determining the type of cell death (necrosis versus apoptosis) mediated by lysosomal pathway seems to be the magnitude of lysosomal permeabilization, and consequently the amount of proteolytic enzymes released into the cytosol (Li *et al.*, 2000; Bursch, 2001; Turk *et al.*, 2002a). Once in the cytosol, lysosomal enzymes can contribute to the execution of the apoptotic program either by direct cleavage of key cellular substrates or by acting in concert with the caspase signalling pathway (Leist and Jaattela, 2001b; Leist and Jaattela, 2001a)(see figure 15).

The precise mechanism by which **lysosomes are permeabilized** is not known even though some hypotheses have been raised. The first one involved **sphingosine.** This component has been proposed to permeabilize lysosomes by its detergent-like action (Kagedal *et al.*, 2001b). Another possible

mechanism of lysosomal permeabilization involves the **generation of Reactive Oxygen Species (ROS)** probably generated by intralysosomal iron-catalized oxidative process (Antunes *et al.*, 2001; Dare *et al.*, 2001; Persson *et al.*, 2003). Experimental evidence suggest that ROS-induced lysosomal permeabilization usually precedes mitochondrial dysfunction (Roberg and Ollinger, 1998; Guicciardi *et al.*, 2000). Finally, some studies have suggested that short term exposure to low concentration of H_2O_2 may induce lysosomal permeabilization indirectly by the **activation of phospholipase A2 (PLA2)** which would cause a progressive destabilization of the membranes of intracellular organelles, including lysosomes and mitochondria by degradation of the membrane phospholipids (Zhao *et al.*, 2000). Another intriguing explanation could be the involvement of the **translocation of pro-apoptotic Bcl-2 proteins inducing permeabilization** in a manner similar to what is observed on mitochondrial level. This hypothesis have been recently confirmed by Kagedal and co-workers (Kagedal *et al.*, 2005) and was also supported by studies demonstrating that the phosphorylated form of Bcl-2 blocks oxidant-induced apoptosis, at least in part by stabilizing lysosomes (Zhao *et al.*, 2000) (Zhao *et al.*, 2001).

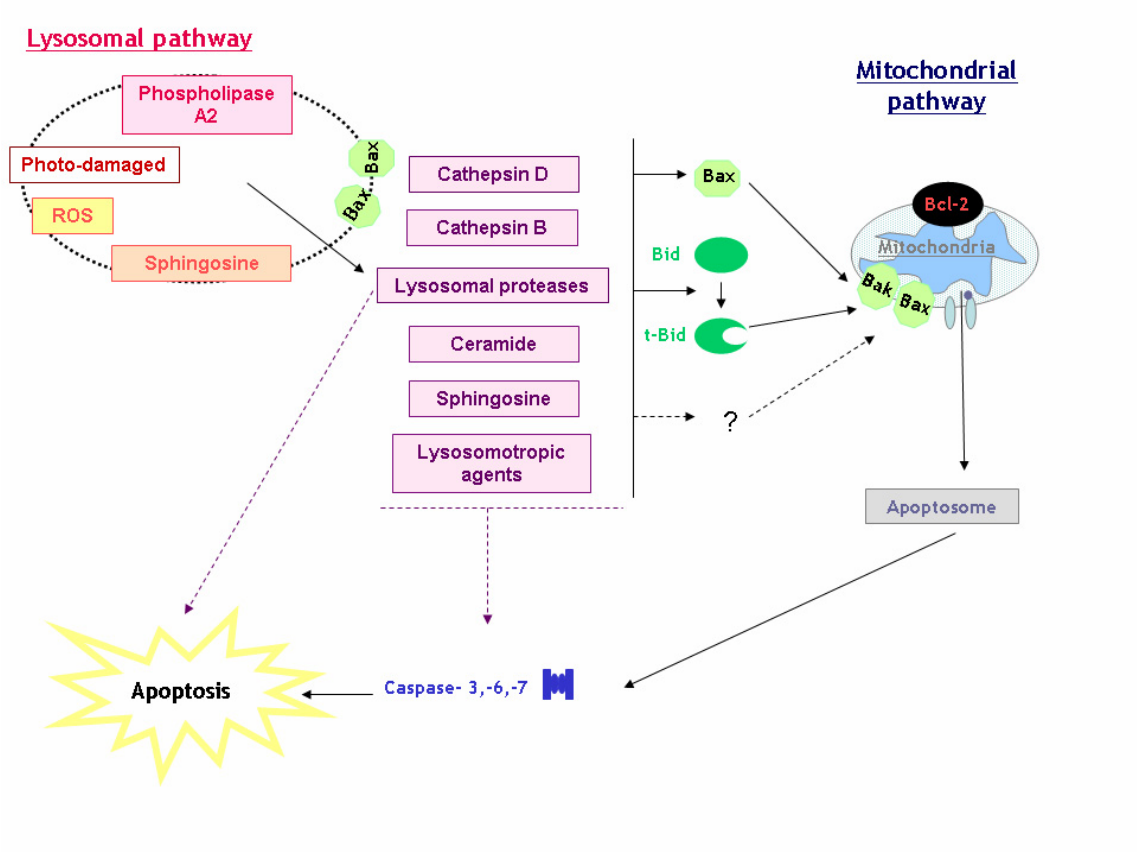


Figure 15: *Lysosomal-mediated death pathway. Lysosomal permeabilization can result from a detergent-like action of sphingosine, the production of ROS probably by the formation of iron-catalized oxidative process, the activation of phospholipase A2 or following insertion of pro-apoptotic proteins such as Bax. This permeabilization allows the release of lysosomal constituents which can activate directly or indirectly effector caspases. The latter one is more probable since mitochondrial pathway is usually activated after lysosomal events.*

Lysosomal permeabilization appears to be an early event in the apoptotic cascade occurring before mitochondrial and caspases activation (Roberg *et al.*, 1999; Li *et al.*, 2000; Bidere *et al.*, 2003; Boya *et al.*, 2003). Thus proteases released from lysosomes seem to be able to trigger the initiation phase of apoptosis. But how do lysosomal cathepsins promote apoptosis after permeabilization? A first possible mechanism is a direct cleavage of caspases (Schotte *et al.*, 1999; Vancompernolle *et al.*, 1998) but it seems more probable that the activation of caspases is obtained by an indirect way such as the cleavage of Bid into truncated-Bid (Stoka *et al.*, 2001; Katunuma *et al.*, 2004). However, a growing body of evidence suggests that lysosomal proteases rather promote apoptosis by acting on mitochondrial pathway. Indeed, the release of

these proteases may cause mitochondrial dysfunction directly (Zhao *et al.*, 2003) or indirectly by activating Bcl-2 proteins such as Bid (Stoka *et al.*, 2001; Cirman *et al.*, 2004) or Bax (Bidere *et al.*, 2003; Boya *et al.*, 2003). Translocation of Bax to mitochondria has been proposed to be mediated by cathepsin D (Boya *et al.*, 2003).

If the way by which lysosomes are permeabilized is still elusive, the involvement of cathepsins, i.e their precise role in the apoptotic signalling, is also controversial. One of the most controversial issues regarding their role outside the lysosomes is their actual ability to function at neutral pH (Turk *et al.*, 1993; Turk *et al.*, 1995; Turk *et al.*, 2000). Among all cathepsins, cathepsin B and cathespin D have been widely involved in several models of apoptosis. Cathespin B has been found to be essential in bile-acid induced hepatocytes apoptosis (Roberts *et al.*, 1997; Jones *et al.*, 1998; Faubion *et al.*, 1999; Canbay *et al.*, 2003), in TNF-α-induced apoptosis of primary hepatocytes and tumor cells (Guicciardi *et al.*, 2000; Guicciardi *et al.*, 2001; Foghsgaard *et al.*, 2001), and after brain ischemia (Yamashima *et al.*, 1998; Tsuchiya *et al.*, 1999). Cathepsin D has been involved in apoptosis induced by staurosporin (Bidere *et al.*, 2003; Johansson *et al.*, 2003), interferon-γ, Fas/CD95/APO-1 and TNF-α (Deiss *et al.*, 1996; Demoz *et al.*, 2002), oxidative stress (Roberg and Ollinger, 1998; Roberg *et al.*, 1999; Ollinger, 2000; Kagedal *et al.*, 2001a), sphingosine (Kagedal *et al.*, 2001b) and p53 (Wu *et al.*, 1998).

c. The Ubiquitin-Proteasome Pathway

The Ubiquitin-Proteasome Pathway (UPP) is the major extra-lysosomal pathway responsible for intracellular protein degradation in eukaryotes. This pathway has been shown to be involved in the regulation of critical cellular processes such as transcription, cell cycle progression, oncogenesis, growth, and development, selective elimination of abnormal proteins and antigen processing (Glickman and Ciechanover, 2002), but also in apoptotic process (Orlowski, 1999). Degradation of proteins by the UPP involves two distinct and

successive steps: (i) covalent attachment of multiple ubiquitin molecules to the target protein, and (ii) degradation of the targeted protein by the 26S proteasome. The 26S proteasome is a 2.1MDa complex composed of the 20S core catalytic complex, which is a cylindrical stack of four seven-membered rings, flanked on both sides by 19S regulatory complexes.

One of the first reports linking the process of ubiquitination with apoptosis emerged from studies of intersegmental muscle programmed cell death in the tobacco hawkmoth *Manducta Sexta* (Schwartz *et al.*, 1990) where Schwartz and colleagues observed an increase in polyubiquitin gene expression. In these dying cells, there was increased ubiquitinating enzymes activity as well as an increase in proteasome components and proteasomal activities (Haas *et al.*, 1995). However later, together with the isolation from *Streptomyces* of the first proteasomal inhibitors, lactacystin which binds and inhibits several proteasomes activities (reviewed in (Fenteany and Schreiber, 1998), controversies appeared since addition of inhibitors were found to be able to induce apoptosis in most of cell lines and sometimes to protect cells from apoptosis in other cell lines. The observation that proteasome inhibitors induce apoptosis in most cells type has particularity contributed to the development of new anti-cancer treatments. Bortezomib represents certainly the first developed compound (Velcade®, formerly PS-341) and is currently under the late phase of clinical development (Jackson *et al.*, 2005; Richardson *et al.*, 2003; Schenkein, 2005; Blade *et al.*, 2005).

Many of apoptosis regulatory proteins including Bcl-2 family proteins such as Bax (Li and Dou, 2000) or Bid (Breitschopf *et al.*, 2000), IAP family proteins (Yang *et al.*, 2000; Yang and Li, 2000; MacFarlane *et al.*, 2002; Martin, 2002) and inhibitors of NF-κB (IκB) (Orian *et al.*, 1995; Kovalenko *et al.*, 2003) have been identified as target substrates for ubiquitination and as being degraded by the proteasome. Thus, **pharmacological inhibition of the 26S proteasome** generally results in **apoptotic cell death** due to the **dysregulation or failure in the degradation of these regulatory proteins.**

The available evidence support that proteasomes act in the apoptotic process upstream of both caspase activation cascade and of mitochondria (see figure 16) (Lang-Rollin *et al.*, 2005; Henderson *et al.*, 2005; Jana *et al.*, 2004; Pei *et al.*, 2003; Jana *et al.*, 2001; Qiu *et al.*, 2000). Furthermore, it has been recently demonstrated that the mitochondrial cell death suppressors carried by human and murine cytomegalovirus confer resistance to proteasome inhibitor-induced apoptosis (McCormick *et al.*, 2005). Where the ubiquitin-proteasome system may really act in the apoptotic signalling may be at the level of the BH3-only proteins. Indeed, several recent reports now suggest that the availability of Bim, Bid or Bik is influenced by the ongoing destruction of these proteins by the proteasome pathway (Ley *et al.*, 2003). This targeting of BH3-only proteins but also other BH-3 pro-apoptotic proteins (Bax, Bak) may therefore play an important role also in regulating the threshold of apoptosis.

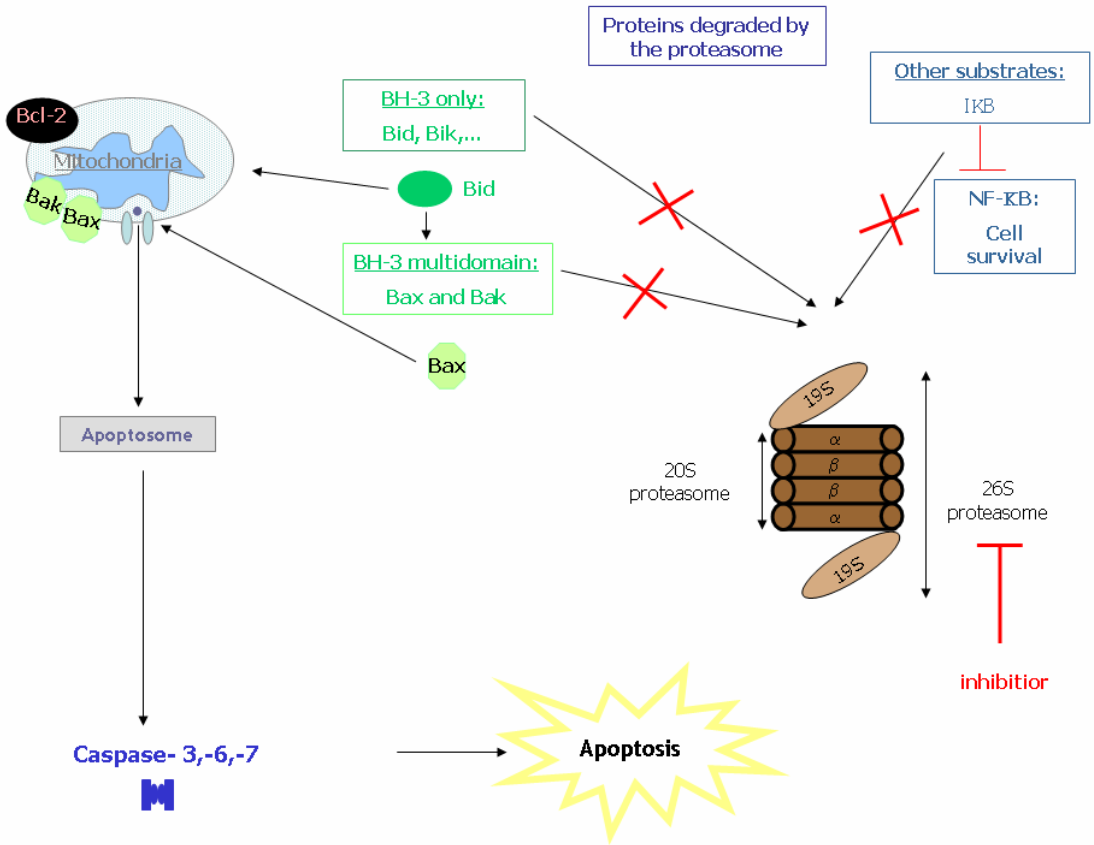


Figure 16: *Inhibition of the proteasome results in accumulation of some of its pro-apoptotic substrates such as Bax. Increase in cellular Bax content create a proapoptotic environment and lead to mitochondrial activation and then cellular death by apoptosis.*

The inhibition of the proteasome may also contribute to the initiation of apoptosis in several other ways than the BH3 protein (reviewed in (Zhang *et al.*, 2004)). Indeed, IκB, the inhibitor of NF-κB, is also degraded by the proteasome and thus an inhibition of its degradation will results in accumulation of this inhibitors preventing the activation of several targert of NF-κB, most of them protecting cells from death (Bcl-2, FLIP, or IAPs) (Wang *et al.*, 1998a). The p53 tumor suppressor is also regulated by the proteasome. Stabilization of p53 as a consequence of DNA damage can then lead to cell cycle arrest and cell death (Li *et al.*, 2002a).

D. Endoplasmic reticulum

The endoplasmic reticulum is another organelle that is a source of proapoptotic initiator signals. Main functions of the ER include protein synthesis and storage of a rapidly mobilized pool of calcium.

Any disturbances detected in the ER trigger an evolutionary conserved response, termed **Unfolding Protein Response (UPR).** The initial intent of the UPR is to adapt to the changing environment and re-establish normal ER function. However, when this adaptation fails because ER stress is prolonged and/or excessive, ER-initiated pathways sound the alarm and trigger cell suicide by apoptosis.

a. *Adaptation to ER stress:*

The ER stress responses of eukaryotic cells consist of three different mechanisms: (1) translational attenuation to limit further accumulation of misfolded protein (Harding *et al.*, 1999); (2) transcriptional activation of genes encoding-ER resident chaperones such as BiP/Grp78 and Grp94 (Gething and Sambrook, 1992), enzymes such as protein disulfide isomerase (PDI) (Dorner *et al.*, 1992); and (3) ER-associated degradation (ERAD) which serves to reduce the stress and thereby restore the capacity by directed misfolded proteins in the ER back into the cytosol for their degradation by the 26S

proteasome (Mori, 2000). The ER stress is intiniated by three type of ER membrane proteins: ATF-6, IRE1 and PERK.

If these adaptatives responses are not sufficient to relieve cells from ER stress, cells undergo apoptosis to destroy the ER stress-damaged cells themselves.

b. The ER stress-induced apoptotic pathways

If ER stress is important and the **Unfolding Protein Response (UPR)** persists, Ire1- and ATF6-dependent expression of transcription factor CHOP (GADD153) together with the continued activation of Ire1 initiate apoptotic signalling. CHOP inhibits expression of some genes while enhancing others (Ubeda *et al.*, 1996). Activated Ire1 associates with TRAF2 and ASK1 to activate caspase-12 and JNK (Urano *et al.*, 2000; Yoneda *et al.*, 2001). Caspase-12 (which can be also activated by calpain) activates caspase-3. Caspase-12 has been described as the specific caspase of the ER (Nakagawa *et al.*, 2000) but also caspase-7 (Rao *et al.*, 2001). However, the relevance of caspase-12 has been questioned since it appears absent in most humans (Fischer *et al.*, 2002). Another caspase has been proposed to be an ER specific caspase and is caspase-4 (Hitomi *et al.*, 2004) although controversies remain about the ER-specificic caspases.

Like other pathways leading to apoptosis, the Bcl-2 family members have been shown to be involved in the ER pathway with similar regulation ability. Some investigations demonstrated that Bax and Bak are involved in the increase of the ER calcium store size, and thus increasing the proapoptotic potential of ER calcium release (Scorrano *et al.*, 2003). Some proteins of the Bcl-2 family have been shown to be more specific to the ER such as for the pro-apoptotic proteins Spike (Mund *et al.*, 2003) or Bik (Yang *et al.*, 1995; Mathai *et al.*, 2002), leading to the BH3-only proteins. Bl-1 (Chae *et al.*, 2003) and BAR (Zhang *et al.*, 2000) are two ER specific proteins that have been shown to interact with Bcl-2 family proteins and inhibit apoptotic signalling.

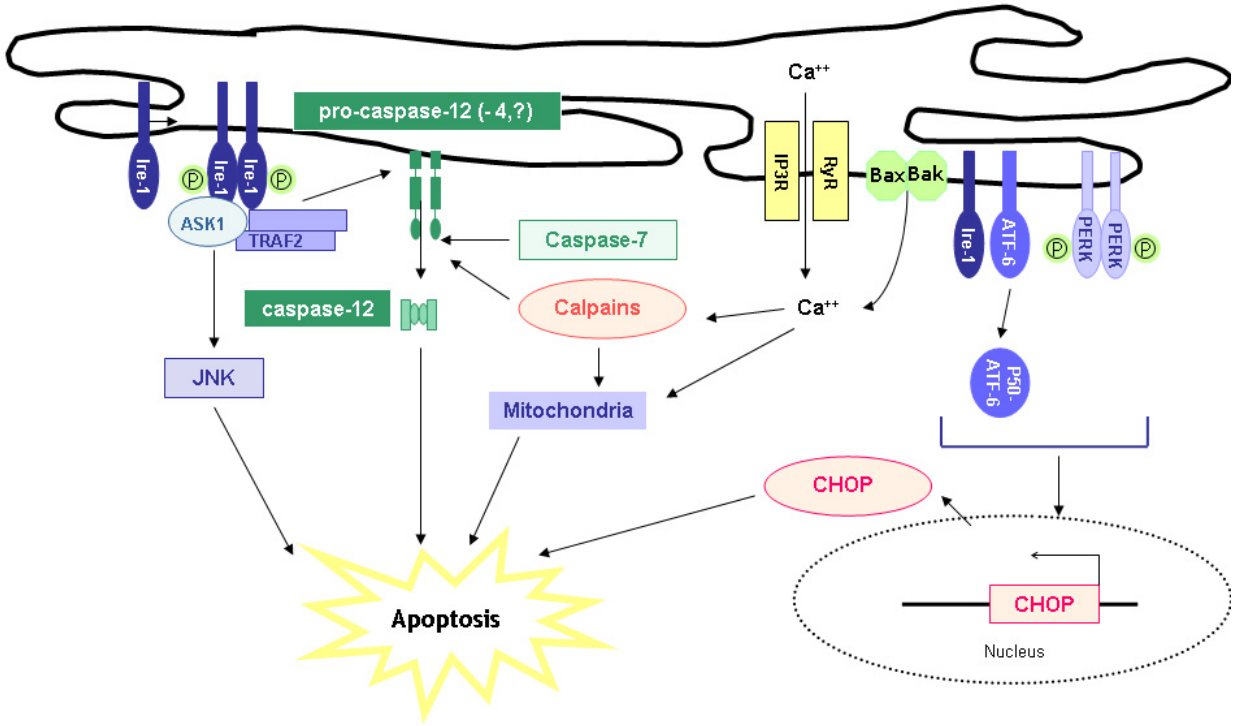


Figure 17: *Mechanisms of apoptosis in response to ER stress. Severe and prolonged ER stress triggers apoptosis through induction of CHOP, activation of caspase-12 and ASK1-JNK pathway and the regulation of the ER homeostasis by Bax/Bak. Calcium release contributes also to the activation of calpain, which in turn can activate caspases.*

Some ER perturbations can inhibit ER calcium pumps (SERCA) and activate calcium release via the inositol 1, 4, 5 triphosphate recetor/ Ca^{++} channel (IP_3) with the consequence that calcium dumps into the cytosol where it can activate apoptosis by many different ways. (1) Calcium released from the ER can be taken up by mitochondria allowing its permeabilization and the release of the proapoptotic components (Szabadkai and Rizzuto, 2004; Orrenius *et al.*, 2003). (2) Calcium release can also activate phospholipase A2 (PLA2) responsible of the ensuing formation of arachidonic acid also able to induce mitochondrial activation (Penzo *et al.*, 2004). (3) Cytosolic calcium can activates calpain proteases involved in pathological cell death (Chan and Mattson, 1999; Huang and Wang, 2001), and whose substrates include Bax and Bid (which are activated) (Wood *et al.*, 1998; Wood and Newcomb, 1999) Bcl-2 and Bcl-xl (which are inhibited) and several caspases (reviewed in (Breckenridge *et al.*, 2003). (4) Calcium could also activate calcium-dependent phospholipids

scramblases such as those responsible for the transfer of phosphatidylserine from the inner leaflet to the outer leaflet of plasma membrane or those responsible for the transfer of cardiolipin from the inner mitochondrial membrane to the outer mitochondrial membrane (McMillin and Dowhan, 2002; Kuwana *et al.*, 2002). (5) Calmodulin activated by calcium induces the phosphatase calcineurin whose role is to dephosphorylate Bad allowing its binding to Bcl-xl (Wang *et al.*, 1999). (6) Calcium could also act by activating calcium-dependent isoform of NO synthase and (7) finally calcium has been shown to activate Drp-1 which has been involved in Bax-mediated release of cytochrome c by regulation of fission process (Szabadkai *et al.*, 2004).

E. Golgi pathway

The Golgi complex plays a central role in processing and sorting of membrane and secretory proteins cargo from the endoplasmic reticulum to the plasma membrane or other post-Golgi destinations. During apoptosis, Golgi stacks disperse and disassemble into tubulo-vesicular clusters (Sesso *et al.*, 1999; Lane *et al.*, 2002; Machamer, 2003) through caspase-mediated cleavage of Golgi structural proteins (Shorter and Warren, 2002; Walker *et al.*, 2004). The Golgi pool of caspase-2, localized to the cytoplasmic face of the Golgi complex (Mancini *et al.*, 2000) could initiate apoptosis upon stress from the secretory pathway (Maag *et al.*, 2005). Golgi caspase substrates include several structural or membrane trafficking proteins of the Golgi: golgin-160 (regulator of Golgi structure), GRASP65 (a stacking protein) and p115 (a vesicle transport protein) (Mancini *et al.*, 2000; Lane *et al.*, 2002; Chiu *et al.*, 2002), giantin (Lowe *et al.*, 2004) and GM130 (Chiu *et al.*, 2002; Walker *et al.*, 2004), the t-SNARE syntaxin-5 (Lowe *et al.*, 2004), the intermediate chain of dynein, and the $p150^{Glued}$ subunit of dynactin (Lane *et al.*, 2002). After some apoptotic stimuli, caspases cleavage of Golgi structural proteins is likely to be the downstream result of effector caspase activation allowing the packaging of Golgi remnants into apoptotic blebs for disposal. However, recent studies have involved cleavage of Golgi proteins in upstream apoptotic signalling events. Indeed,

Maag showed that the expression of non-cleavable golgin-160 prevents drug-specific cleavage of endogenous golgin-160 due to a block in upstream caspase activation (Maag *et al.*, 2005).

In conclusion, the induction of these different pathways is dependent of the stimulus but also probably of the cellular and tissular nature. It is thus of critical importance before designing new targets and therapeutics to analyze the precise role played by apoptosis in a particular cell type but also if this particular cell type has specific regulators or pathway leading to apoptosis. The following section resumes what is known in this context for the kidney, the different pathways that could be induced by nephrotoxins but also some of promising approach to reduce nephrotoxin-induced apoptosis in the kidney.

4. APOPTOSIS IN THE KIDNEY

Apoptosis plays a critical role during nephrogenesis, but also later to maintain tissular homeostasis. As any physiological process, a decrease or an increase of renal cell apoptosis can be associated with some pathologic conditions (such as polycystic kidney disease, renal cancer or diabetic nephropathy) or with toxic insults caused by drugs, metals or toxins.

4.1. Apoptosis during kidney development

As well as cell differentiation and morphogenesis, normal kidney development involves a strict control of proliferation and apoptosis (Coles *et al.*, 1993; Winyard *et al.*, 1996). There is a distinct temporal pattern of apoptotic deletion during rat kidney development. Approximately 2.7% of cells within the nephrogenic zone are apoptotic during the embryonic period, this percentage declines to a basal level of 0.15% by the fourteenth postnatal day (Coles *et al.*, 1993). A second peak of apoptosis occurs in the medullar papilla at postnatal days 6-7 when the percentage of apoptotic cells reached 3.2%. Peak apoptosis in the medullar papilla correlates temporally with deletion of the thick ascending limb cell during the formation of the ascending thin limb of the loop of Henle (Kim *et al.*, 1996).

Some apoptosis regulators have been shown to be crucial for kidney development. Among these factors, **Bcl-2** which has anti-apoptotic properties, seemed to play an important role. Indeed, mice deficient in Bcl-2 (Bcl-2 -/-) develop renal hypoplasia/cystic dysplasia. Kidneys in these mice undergo fulminant apoptosis of the metanephric blastema during early embryogenesis and the renal epithelial cells do not complete terminal differentiation in the postnatal kidney (Sorenson and Sheibani, 1999; Sorenson *et al.*, 1995; Sorenson and Sheibani, 2002). Moreover, during nephrogenesis, apoptosis tends to be inversely correlated with Bcl-2 expression (Chandler *et al.*, 1994). Its expression is high early in the embryonic kidney and its level decreases

significantly at later times so that its expression is normally low in the postnatal kidney (Novack and Korsmeyer, 1994). During development, Bcl-2 is expressed in the ureteric bud (Novak 1994 61) and in epithelial condensates of the metanephric blastema (Chandler *et al.*, 1994). However, Bcl-2 is not expressed in uninduced mesenchyme (Chandler *et al.*, 1994). During differentiation of the renal vesicle, the epithelium of the nascent glomerulus is initially strongly positive, but upon maturation and vascularization, staining becomes limited to the parietal layer of the Bowman's capsule. Initially, tubules are strongly positive for Bcl-2, but upon maturation staining significantly decreases. The collecting duct has faint staining for Bcl-2 and renal calyces are negative (Chandler *et al.*, 1994). In the adult human, expression is significantly lower but detected in the parietal epithelium of Bowman's capsule, the distal convoluted tubule, the loop of Henle, the proximal tubules and papillary collecting ducts (Chandler *et al.*, 1994). At the contrary, pro-apoptotic **Bax** is rather widely expressed in adult kidney (Krajewski *et al.*, 1994).

How about caspases during renal development? **Caspase-1, -2, and -3** together with another apoptosis-inducer protein **Apaf-1** are expressed constantly in the kidney of mice from E12 (early embryogenesis, 12 days of gestation) to neonates (Hayashi and Araki, 2002). There is no difference in expression levels of caspase members throughout this period. However, expression of caspase-2 seems to be increased after birth, while the other caspases do not show significant changes during kidney development. Double straining TUNEL-Caspase-3 of the metanephros clearly shows that caspase-3 is present in apoptotic cells, suggesting that this caspase may play an important role during renal development (Hayashi and Araki, 2002). The addition of caspase-3 and caspase-9 respective inhibitors significantly suppressed metanephroi growth. The inhibitor of caspase-1 did not change metanephric growth, supporting that the mitochondrial pathway is important for kidney development (Hayashi and Araki, 2002).

In conclusion, apoptosis and thus regulators of the apoptotic machinery seem to play an important role during renal development. Thus, the predicted effect of renal toxicant exposure *in utero* will depend on the cellular events during that developmental period and whether the toxicant induces or inhibits apoptosis. An inducer of apoptosis may kill cell populations that are required for normal tubular functions whereas an apoptosis inhibitor may result in malformed tubules constituted of cells with a poor differenciated phenotype.

4.2. Apoptosis induced by nephrotoxic agents

If drugs can be harmful by interfering with the normal development of the kidney, they can also execute their toxic effect by modifying apoptosis in the mature kidney, and this is all the more because kidney is particularly susceptible to drugs and toxics. This particular susceptibility can be attributed in part to its unique anatomic and physiologic features: kidney receives 25% of the resting cardiac output, and many substances are freely filtered across the glomerular capillary wall into the urinary space. The surface area of the renal vasculature and tubules is very large, thus providing ample opportunity for exposure to toxins. Many circulating compounds are also taken up by transporters into tubule epithelial cells and secreted into the lumen. The concentration within the kidney may be increased further by the countercurrent exchange system that results from the unique parallel arrangement of tubules and vasculature within the renal medulla.

Furthermore, kidney is an important site of elimination of drugs and xenobiotics, so it contains several mechanisms for the handling and the excretion of these compounds (see figure 18). For example, aminoglycosides are excreted mainly by glomerular filtration and then they access the lumen of proximal tubules where they can interact with anionic phospholipids of the brush border membrane of proximal tubular cells. This binding allows them to access megalin, the receptor that mediates their reabsorption. Megalin also mediates the reabsorption of other essential components such as calcium, lipoproteins,

vitamin-carrier complexes and contributes to the renal clearance and uptake of polybasic drugs such as aprotinin. After their endocytosis, aminoglycosides are then transported from the endosomes to the lysosomes where they are sequestered due to their polar state in an acidic environment. Their accumulation in proximal tubular cells is responsible of their toxicity and as mentioned earlier this has been associated to their ability to induce cell death by apoptosis (El Mouedden *et al.*, 2000b; El Mouedden *et al.*, 2000a).

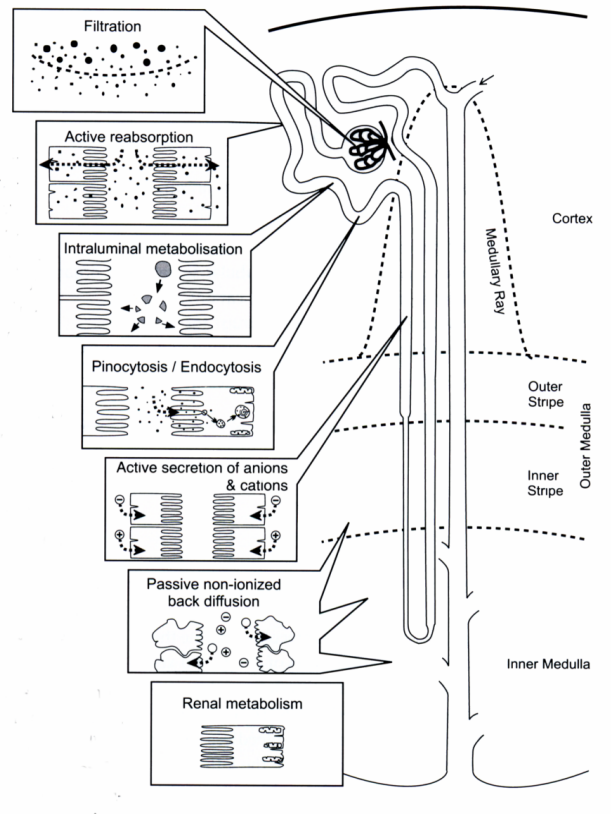


***Figure18**: Illustrations of the main localisation and cellular handling of drug and xenobiotics. From Roch-Ramel and De Broe. (Roch-Ramel F. and M.E.De Broe, 2003).*

Apoptosis has been shown to play a prominent role in the development of acute renal failure and this observation has been validated both in animal models and in clinical kidney diseases (Olsen *et al.*, 1985; Shimizu and Yamanaka, 1993; Ortiz *et al.*, 2000; Ortiz-Arduan *et al.*, 1996) but has been also supported by the observation that the expression or activity of apoptosis regulators are modified during acute renal failure in renal cultured cells and in kidney disease (reviewed in (Ortiz *et al.*, 2002)). In this context, numerous nephrotoxic substances from

very diverse origin have now been shown to be able to induce apoptosis in different part of the kidney (see table 1): among these substances antibiotics such as aminoglycosides or fluoroquinolones, cytostatic drugs such as cisplatin or doxorubicin, immunosuppressors such as cyclosporins, diuretics such as the thiazides but also the radio-contrast agents or the non-drugs such as heavy metals (Cadmium) or toxins (Ochratoxin A). For the majority of the nephrotoxic substances, their toxic manifestations have been associated to their ability to induce apoptosis underlining the importance of such cell death in renal injury.

Table 1 resume drugs and toxicant inducing apoptosis in kidney or renal cells. (from a review in preparation: How to modulate renal apoptosis induced by nephrotoxic drugs: cellular and molecular approaches. H. Servais,[a] O. Devuyst,[b] P.M. Tulkens[a] and M.-P. Mingeot-Leclercq. [a])

DRUGS OR TOXIC SUBSTANCES INDUCING APOPTOSIS IN THE KIDNEY		
Cisplatin	Mouse proximal tubular cells Mouse collecting duct cells	(Arany *et al.*, 2004)– (Takeda *et al.*, 1997; Vickers *et al.*, 2004) (Takeda *et al.*, 1998)
Doxorubicin	Rat: tubular epithelial cell and distal tubule cells	(Zhang *et al.*, 1996)
Zoledronate	Human: tubular epithelial cells	(Markowitz *et al.*, 2003)
Cyclosporine	Rat: tubular and interstitial cells Cultured Madin Darby Canine Kidney cells (MDCK)	(Thomas *et al.*, 1998) (Sheikh-Hamad *et al.*, 2001)
Rapamycin	Cultured Mouse Proximal Tubular cells (MPT)	(Lieberthal *et al.*, 2001)
Statins	Cultured murine tubular cell	(Blanco-Colio *et al.*, 2002)
Radio-contrast agents	Cultured Madin Darby Canine Kidney cells (MDCK)	(Hizoh *et al.*, 1998) (Hizoh and Haller, 2002)
Thiazides	Rat: distal convoluted tubular cells	(Loffing *et al.*, 1996)
Diclofenac	Mice: proximal and distal tubular cells	(Hickey *et al.*, 2001)
Paracetamol	Cultured murine proximal tubular cells	(Lorz *et al.*, 2004)
Cidofovir	Human tubules Cultured human proximal tubular epithelial cells (HK-2)	(Ortiz *et al.*, 2005)

Amphotericin B	Cultured Proximal tubular cells from pig (LLC-PK1) Cultured Medullary Interstitial Cells (RMIC) Rat	(Varlam *et al.*, 2001)
Ciprofloxacin	Human: distal tubular cells	(Dharnidharka *et al.*, 1998)
Aminoglycosides	Cultured Madin Darby Canine Kidney cells (MDCK) Rat: Proximal Tubular Cells Cultured Proximal tubular cells from pig (LLC-PK1)	(El Mouedden *et al.*, 2000b) (El Mouedden *et al.*, 2000a) (Servais *et al.*, 2005)
Mercuric chloride	Cultured rat proximal tubular cells (WKPT) Cultured Proximal tubular cells from pig (LLC-PK1)	(Thevenod, 2003) (Duncan-Achanzar *et al.*, 1996)
Cadmium	Cultured rat proximal tubular cells (WKPT)	(Thevenod, 2003)
Oxalate	Cultured Madin Darby Canine Kidney cells (MDCK	(Cao *et al.*, 2004)
Endotoxins	Mice (Fas-/-, TNFR1-/- TNFR2-/-) C3H/HeJ Mice Human tubular epithelial cells Human and Mouse: tubular cells	(Cunningham *et al.*, 2002b) (Karpman *et al.*, 1998)
Ochratoxin A	Primary Rat Kidney cells (PRK) Cultured Cells: Opossum Kidney (OK) and Normal Rat Kidney Epithelial (NRK-52E)	(Kamp *et al.*, 2005) (Sauvant *et al.*, 2005)
Microcystin	Rat: kidney cortex and medulla	(Milutinovic *et al.*, 2003)
Dichloroacetic acid	Rat: proximal tubules	(Lantum *et al.*, 2002)
3,4 dideoxyglucosone-3ene	Cultured murine proximal tubular epithelial cells (MCT)	(Justo *et al.*, 2005)

Table 1: *Different molecules that have been shown to induce apoptosis in the kidney. (from a review in preparation: How to modulate renal apoptosis induced by nephrotoxic drugs: cellular and molecular approaches. H. Servais,[a] O. Devuyst,[b] P.M. Tulkens[a] and M.-P. Mingeot-Leclercq.[a])*

The way by which all these toxics and drugs can induce apoptosis is currently widely studied. Table 2 resumes the molecular pathway(s) that have been described for the different toxic and drugs inducing apoptosis in the kidney (the different pathways leading to apoptosis have been described in the section 3.2 of this introduction). From a general point of view, mitochondrial pathway seemed to be involved in numerous models of toxic-induced apoptosis whereas more specific pathways such as endoplasmic reticulum and lysosomal pathways are less frequent. However, these results should be analyzed with caution since there is still an incomplete understanding of the molecular

regulation of apoptosis in a general point of view but also more specifically in the kidney. Furthermore, with the progressive understanding of apoptotic process, it appears that the diverse nephrotoxic substances induce apoptosis in a stimulus- and probably cell-specific manner. The involvement of mitochondria in most cases of nephrotoxins-induced apoptosis could certainly be attributed to the fact that mitochondria seemed now to play a more central regulatory role in apoptosis, the initiating apoptosis event seemed to be more specific to a drug and thus initiated by very diverse pathway involving either the lysosomes, or the endoplasmic reticulum, or the golgi or other cellular constituents. The identification of the initiating stress induced by a given substances is of critical importance for the development of specific and accurate therapeutic intervention.

MOLECULAR PATHWAYS INVOLVED IN DRUGS-INDUCED APOPTOSIS IN THE KIDNEY				
Primary subcellular organites	Drugs	Evidences	Models	References
Mitochondria	Cisplatin	Release of Omi from mitochondria to the cytoplasm and degradation of XIAP	Primary mouse proximal tubule cells	(Cilenti *et al.*, 2005)
		Effect of mitochondrial blockade	Renal collecting duct-derived cells (MDCK-C7)	(Schwerdt *et al.*, 2005)
		Decrease in relative ratio of Bcl-2/Bax in the outer medulla	Rat kidney	(Sheikh-Hamad *et al.*, 2004)
		Release of Cytochrome c	Primary cultured rabbit proximal tubules	(Baek *et al.*, 2003)
		Mitochondrial permeability transition	Murine renal proximal tubular epithelial mProx cells	(Satoh *et al.*, 2003)
		Activation of Bax Mitochondrial permeability transition Release of cytochrome c Activation of caspase 9	LLC-PK1	(Park *et al.*, 2002)
		Overexpression of MnSOD	Human embryonic kidney 293 (HEK293)	(Davis *et al.*, 2001)
		Bad phosphorylation Activation of caspase 9	LLC-PK1	(Kaushal *et al.*, 2001)
		Translocation of endogenous Bax from the cytosolic to the membrane fractions Release of cytochrome *c*	Mouse collecting duct cells	(Lee *et al.*, 2001)
		Inhibition of complexes I to IV of the respiratory chain.	LLC-PK1	(Kruidering *et al.*, 1997)
		Inhibition of Na^+/K^+ ATPase Collapse of the mitochondrial membrane potential	Renal proximal tubular cells	(Kuhlmann *et al.*, 1997)
	Contrast Agents	Decrease in relative ratio of Bcl-2/Bax	SHR Rats	(Zhang *et al.*, 1999)
	Cyclosporin A	Decrease in relative ratio of Bcl-2/Bax	Male Sprague-Dawley rats	(Lee *et al.*, 2004)
		Translocation of Bax to the mitochondria Release of cytochrome c and Smac/Diablo Loss of mitochondrial membrane potential. Activation of caspase-9	Murine proximal tubular epithelial	(Justo *et al.*, 2003)

	Gentamicin	Loss of mitochondrial membrane potential. Activation of caspase-9 Release of cytochrome c	LLC-PK1	(Servais *et al.*, 2005)
	G418	Release of cytochrome c	Rat Kidney Cells	(Jin *et al.*, 2004)
	Statins	Decrease of Bcl-xL/Bax ratio Release of cytochrome c Activation of caspase 9	Murine tubular cells	(Blanco-Colio *et al.*, 2003)
Endoplasmic reticulum	Acetominophen	Upregulated expression of GADD 153 Caspase-12 cleavage	MCT cells (cultured line of proximal tubular epithelial cells harvested originally from the renal cortex of SJL mice)	(Lorz *et al.*, 2004)
	Cisplatin	Increased the activity of ER-iPLA(2)	Rabbit renal proximal tubule cells (RPTC)	(Cummings *et al.*, 2004a)
	Cyclosporin	Induction of GADD 153 expression	Murine proximal tubular epithelial	(Justo *et al.*, 2003)
	G418	Increase in Ca^{2+} concentration Cleavage of m-calpain and procaspase-12	Rat kidney cells	(Jin *et al.*, 2004)
	Tunicamycin	Decrease of apoptosis in renal proximal tubular epithelium in GADD 153 -/- Mice	GADD 153 -/- Mice	(Zinszner *et al.*, 1998)
Lysosomes	Gentamicin	Release of acridine orange Lysosomal permeabilization	LLC-PK1	(Servais *et al.*, 2005)
	Iodinated contrast media (iobitridol and iohexol)	Prominent lysosomal alteration of the proximal convoluted tubular cells	Rats	(Tervahartiala *et al.*, 1997)

***Table 2**: Apoptotic pathways involved in drugs-induced apoptosis. (from the review in preparation: How to modulate renal apoptosis induced by nephrotoxic drugs: cellular and molecular approaches. H. Servais,[a] O. Devuyst,[b] P.M. Tulkens[a] and M.-P. Mingeot-Leclercq.[a])*

4.3. Strategies to decrease cell death-induced by nephrotoxic agents

The pathogenic role of apoptosis described after administration of nephrotoxic drugs has brought apoptosis regulators as key targets for the design of new therapeutic strategies. Of course, all approaches for limiting apoptosis must be applied with consideration for the multiple cell death pathways that exist and the interconnectivity that links the different apoptotic pathways.

4.3.1. **Decreasing drug accumulation in the kidney**

Reduction of the accumulation of toxic drugs would represent one of the most rational approaches to reduce both nephrotoxic potential and apoptosis responses. This strategy has been largely documented in experimental studies. However, only one type of molecules has reached the preclinical stage: they consist of specific antagonists of megalin. Indeed, protection afforded by reducing the uptake of aminoglycosides in the renal cells has been widely documented in experimental models (Takamoto *et al.*, 2005; Schmitz *et al.*, 2002; Watanabe *et al.*, 2004). They have been developed by RecetIcon Laboratories (htpp://www.receptcon.com) to reduce nephrotoxicity of aminoglycosides. These antagonists are currently in the late stage of preclinical trial and will soon enter in phase I.

4.3.2. **Regulating the molecular pathways and pro- or anti-apoptotic factors**

A. Modulation of the protein of the Bcl-2 family:

Due to the central role of mitochondria in apoptosis, regulation of Bcl-2 family proteins constitutes an attractive approach that is currently largely explored in preclinical studies to modulate the cell death in different pathological conditions such as cerebral infarct, fulminant hepatitis, brain ischemia or ischemia/reperfusion injuries in the heart (Cao *et al.*, 2002; Akhtar *et al.*, 2004; Tanaka *et al.*, 2004; Weisleder *et al.*, 2004). Among the molecules that can be promising, minocycline is currently under clinical phase I (Danbury Pharmacal) and has been approved by the FDA for the treatment of Huntighton disease. On experimental model, this molecule has been shown to protect kidney cells from apoptosis induced by different type of insult (Wang *et al.*, 2004) and should constitute a promising to reduce apoptosis in the kidney.

B. Modulation of ROS production

Since ROS generation might be responsible for lysosomal and mitochondrial permeabilization (see earlier), this pathway constitutes another critical target to modulate renal apoptosis. Among promising molecules, edaravone, a free radical scavenger developed by Mitsubishi-Tokyo and currently under Phase III, has been approved for the acute therapy of embolic stroke by the Japanese Ministry of Health, Labor and Welfare in 2001 (Sueishi *et al.*, 2002; Satoh *et al.*, 2003; Iguchi *et al.*, 2004). Idebenone, another molecule developed by Mitsubishi-Tokyo is currently under phase I for the treatment of Friedreich ataxia.

As scavenging of ROS has been reported to protect tubular epithelium from cell death induced by numerous nephrotoxic compounds (Baek *et al.*, 2003) especially by gentamicin and cisplatin (Kuhlmann *et al.*, 1997; Basnakian *et al.*, 2002; Shirwaikar *et al.*, 2003), the protective effect of these scavengers could be interesting to overcome nephrotoxicity.

C. Modulation of the receptor-mediated pathway

Infiltrating leukocytes such as macrophages induce renal cell apoptosis by the release of TNF-α or Fas. One therapeutic approach to reduce such apoptosis could be the use of neutralizing antibodies of TNF-α. Some of them are currently under clinical development: Adalimumab (HUMIRA) from Abbott Laboratories has been approved by the FDA for the treatment of rheumatoid arthritis, psoriasis, ankylosis spondylitis and Crohn disease. Infliximab (REMICADE) developed by Centocor and Shering-Plough has been approved by the FDA to treat Crohn disease and rheumatoid arthritis. Another way to neutralize the effect of TNF-α is by antisense oligonucleotide; it has been proposed by Isis with its molecule ISIS104828, which has been approved by the FDA for the treatment rheumatoid arthritis, psoriasis and Crohn disease. These molecules could represent interesting tool since the inhibition of death receptor signalling (Kelly *et al.*, 2004; Ramesh and Reeves, 2004) or the genetic deletion

or inhibition of TNF-alpha or TNFR2 receptors (Falk *et al.*, 2005) have been shown to be promising approach to reduce apoptosis induced by nephrotoxic drugs.

D. Modulation of caspase-dependent process

The approach of direct caspase inhibition have been extensively explored (Shiozaki and Shi, 2004; Lavrik *et al.*, 2005). Among the promising drugs, we find MX-1013 (developped by Maxim Pharmaceuticals, Inc), a dipeptidyl pan-caspase inhibitor (z-VD-fmk) characterized by a high cell permeability and specificity. This inhibitor has been shown to protect cells from a variety of apoptotic insults and to be active in a number of animal models of apoptosis (Yang *et al.*, 2003). MX 1122, (2,4dichloro-cbz-VD-fmk), a parent compound of MX-1013, yielded also attracting results in preclinical studies (Cai *et al.*, 2004). Yet, an inhibitor of caspase-3 and –7, developed by IDUN Pharmaceuticals, Inc (IDN-8050), has been described to reduce tubular apoptosis in autosomal dominant polycystic kidney disease in short-term studies (Tao *et al.*, 2005). Taken together, these studies suggest that drugs blocking caspase activity may be able to halt or reduce renal apoptosis (Daemen *et al.*, 2002; Cummings *et al.*, 2004b). However, although caspase inhibitors tested in preclinical stages have given promising results, they should be analyzed with caution. Indeed, most of these studies have been driven by the often erroneous belief that caspase would generally dictate the point of “no-return” of cell death. In the context, cells often do not die by apoptosis but succumb to a delayed cell death (Chipuk and Green, 2005; Kroemer and Martin, 2005) that can have an apoptosis-like morphology (with peripheral chromatin condensation yet without karyorrexhis) (Susin *et al.*, 2000) or manifest as autophagic cell death (with accumulation of autophagic vacuoles) (Yu *et al.*, 2004) or necrotic death (with swelling of cytoplasmic organelles and plasma membrane) (Hirsch *et al.*, 1997). This has been observed in apoptosis-induced by cyclopsorin A and acetaminophen where the pan-caspase inhibitor z-VAD-fmk have been shown to prevent apoptosis in both model but fail to maintain cell viability in the long

term (Ortiz *et al.*, 1998). Moreover, a proof-of-principle experiment illustrating that the conditional or tissue specific knock-out of one or several caspases genes can confere true cytoprotection is still elusive.

E. Modulation of serine protease Omi/HtrA2

Omi/HtrA2 is localized in the mitochondria and is released upon mitochondria activation. Its role is to antagonize the XIAP, the later one inhibits processed caspase-9 and processed caspase-3 and -7 with low nanomolar affinity (Takahashi *et al.*, 1998; Shi, 2002). The Omi/HtrA2-specific inhibitor, ucf-101 inhibits the nephrotoxic, apoptotic effect of cisplatin in mice (Cilenti *et al.*, 2005). This inhibitor is currently at preclinical stage and its efficacy in reducing infarct size after myocardial ischemia in mice has also been demonstrated (Liu *et al.*, 2005).

4.3.3. Stimulating the survival pathway and regulating the cell cycle

A. Administration of factors that stimulate the intracellular survival pathway

The cell microenvironment usually contains multiple survival and lethal factors. The potential for interaction between these factors varies in a stimulus- and cell-specific manner. The unravelling of the complex growth factors/cytokines network in kidney (Imai and Isaka, 2002; Ramesh and Reeves, 2004) may lead to novel strategies to prevent apoptosis induced by nephrotoxic drugs. Several studies reported that the absence of survival factors predisposes renal tubular epithelial cells to death induced by lethal cytokines and nephrotoxic drugs (Ortiz *et al.*, 2000). Moreover, nephropathies like immunoglobulin A one, decrease endothelial growth factor synthesis in relation to apoptosis (Coppo and Amore, 2004). The major survival factors for tubular epithelial cells are EGF, IGF-1 (insulin-like growth factor 1), and HGF (hepatocyte growth factor) (Ortiz *et al.*, 2000).

The growth factors inhibit apoptosis by downregulating proapoptotic pathways and stimulating survival promoting pathways such as that involving phosphoinositide 3'-hydroxykinase (PI3K) and the protein kinase Akt (Gibson *et al.*, 1999; Okubo *et al.*, 1998).

a. EGF (epidermal growth factor)

EGF is a renotrophic growth factor constitutively expressed in the distal convoluted tubules and in the thick ascending limb of Henle (Jung *et al.*, 2005). It is involved in recovery from renal injury and protection from apoptotic cell death induced by TRAIL, a member of the death receptor ligand family or unilateral ureteral obstruction in neonatal rats (Gibson *et al.*, 2002; Kiley *et al.*, 2003). Exogeneous EGF also accelerates renal tubular cell regeneration after ischemia (Humes *et al.*, 1989) or exposure to nephrotoxic drugs or toxics like mercuric chloride (Coimbra *et al.*, 1990). The mechanism underlying these salutary effects is largely unknown but signalling pathways converging at Bad phosphorylation could be key factors as demonstrated for growth factor-mediated attenuation of stress-induced apoptosis in vitro and in vivo (Kiley *et al.*, 2003).

b. IGF-1 (insulin-like growth factor 1)

Administration of IGF-1 in murine renal ischaemia/reperfusion injury has comparable effects on apoptosis as caspase inhibition (Daemen *et al.*, 1999). Furthermore, IGF-1 inhibit apoptosis induced by amphotericin B (Varlam *et al.*, 2001) and cidofovir (Justo *et al.*, 2005) and improve outcome in obstructive nephropathy (Chevalier *et al.*, 2000). In vivo, antiapoptotic signalling by IGF-1 is mediated by the PI 3-kinase pathway (Daemen *et al.*, 1999) probably converging at Bad phosphorylation (Kiley *et al.*, 2003).

c. HGF (hepatocyte growth factor)

HGF, has also been reported to protect and salvage from renal ischemic injury (Ernst *et al.*, 2001) as a renotropic and antifibrotic factor. *In vitro* and *in vivo* studies have shown the beneficial effect of administration of HGF on preventing apoptosis in renal tubule cells induced by cisplatin and mercury chloride (Liu *et al.*, 1998) but also by cidofovir (Justo *et al.*, 2005). Moreover, electroporation-mediated HGF gene transfer ameliorated cyclosporine nephrotoxicity, probably by enhancing the phosphorylation of Akt and Bcl-2 (Mizui *et al.*, 2004).

B. Cell cycle regulation

Cell cycle arrest at G1/S or G2/M phase in coordination with enhanced DNA repair may contribute to decrease of apoptosis. So, sodium arsenite, has beneficial effects on cisplatin–induced acute renal failure, by increasing expression of p27, PCNA, and GADD 153 and downregulating expression of cyclin B1 and cyclin D1 (Zhou *et al.*, 2004). In addition the kinase inhibitors p21 and roscovitine, a pharmacological inhibitor of cyclin-dependent kinases, protected from cisplatin-induced cell death even this protection is not merely dependent on cell cycle inhibition (Price *et al.*, 2004). Yet, inhibiting CDK2 activity decreased apoptosis in growth factor-deprived mesangial cells (Hiromura *et al.*, 1999).

4.3.4. Gene therapy

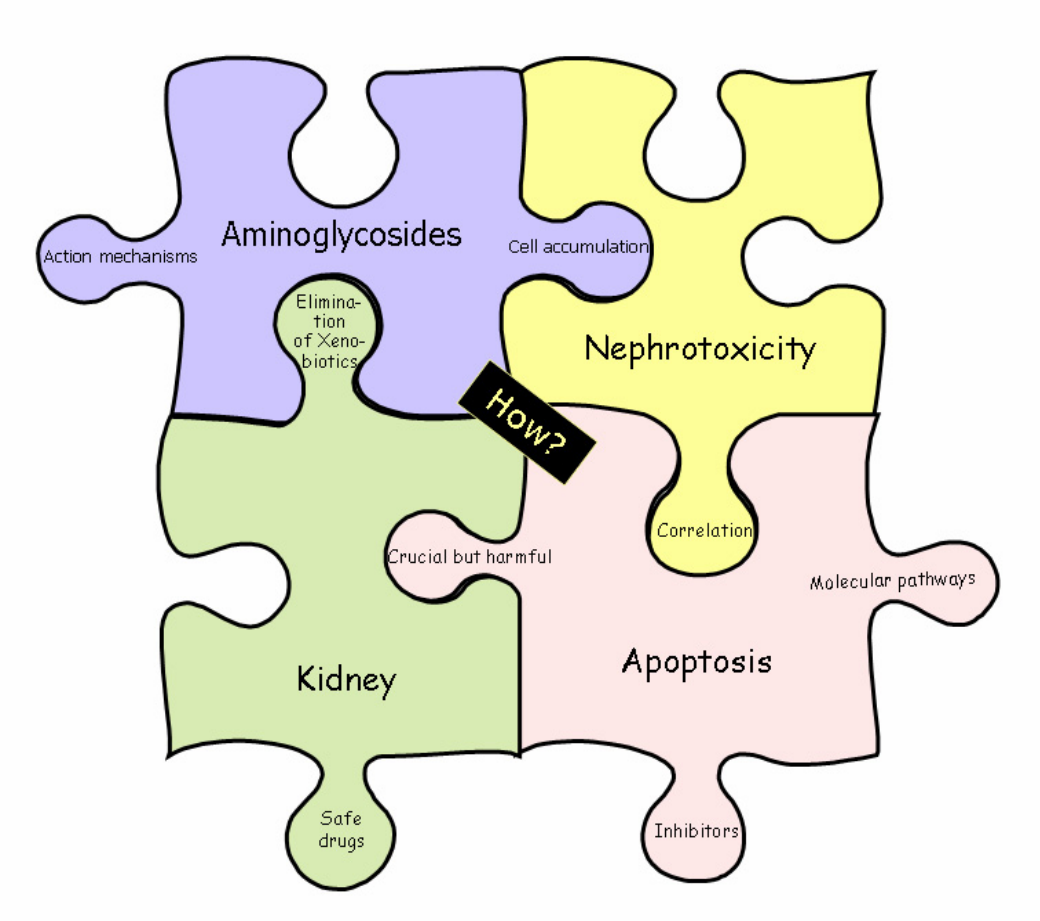
2-D gel-based proteomics and microarray analysis of animal disease models or of animal drug nephropathy models (cisplatin; (Huang *et al.*, 2001)) are powerful approaches for understanding the gene expression programs that underline drug induced apoptosis or pathologies related to apoptosis. So, 2-D gel-based proteomics have been used to characterize the nephrotoxicant, gentamicin (Charlwood J. *et al.*, 2002), cyclosporine (Aicher *et al.*, 1998) or cisplatin (Huang *et al.*, 2001; Bandara *et al.*, 2003). Changes in gene expression in the kidney of rats treated with cisplatin (Amin *et al.*, 2004; Vickers

et al., 2004; Thompson *et al.*, 2004) or rats treated with gentamicin (Amin *et al.*, 2004; Kramer *et al.*, 2004) have also been determined by microarray analysis.

Studies in renal tubular epithelial cells have demonstrated the feasibility and effectivity of gene transfer strategies to promote the expression of Bcl-2 or BclxL and prevent tubular epithelium apoptosis (Takeda *et al.*, 1997; Ortiz *et al.*, 2000). Gene therapies in renal disease reported to date are described in Imai and Isaka (Imai and Isaka, 2002). In particular, genes encoding the NaCl cotransporter (NCC) of the distal convoluted tubule or the Na-K-2Cl cotransporter (NKCC2) of the tick ascending limb could be promising target for treatment of Gitelman or Bartter syndromes respectively (Miyamura *et al.*, 2003; Sabath *et al.*, 2004). The major challenge for physiologists of the beginning post-genomic era, however, is to determine the functions of all genes related to the control of apoptosis and cell division under different pathophysiological conditions (Soutourina *et al.*, 2005).

In conclusion, the intracellular components of the apoptosis cascade are now being unravelled to reveal a wide array of cellular factors and complex pathways converging in programmed cell death. As we gain better insights into the molecular mechanisms behind this cascade, better strategies will be developped for controlling the cell-death responses. The challenges remain to prevent cross-talk between different pathways, to control the cell-specificity, and to define the optimal time frame for therapeutic intervention. Special consideration should be given to take advantage of the presence of specific transporters in renal cells and to operate an appropriate patient selection. Resolution of all these challenges might allow incorporating promising preclinical findings into actual therapeutic practice.

Chapitre II: Aim of the study and elements that have led to this work



Since their discovery in 1940s, aminoglycosides have been widely used for the eradication of infectious agents and they currently still constitute important agents used to treat severe infections. Even if they are now well-known agents, the mechanisms responsible for their nephrotoxicity have long remained unexplained. Indeed, as described in the first part of this manuscript, numerous alterations were associated with nephrotoxicity of aminoglycosides, most of them observed in proximal tubular cells of the kidney but none of them was unambiguously related to toxicity. Between these alterations, the major ones occurred in the apical and basolateral membranes, the lysosomes (development of a phospholipidosis) and various other subcellular components.

First approaches to identify the mechanisms underlying nephrotoxicity had often to use large amounts of the drug administered to animals (usually ten-times the therapeutic ones) in order to observe clear-cut acute tubular necrosis and concomitant kidney dysfunction. The observations by El Mouedden and co-workers that administration to rats of low dosage of aminoglycosides more relevant to clinical situations (gentamicin, amikacin, netilmicin and isepamicin) are able to induce a particular form of cell death, named apoptosis has allowed pieces of puzzle to begin to fall into place (El Mouedden *et al.*, 2000a).

Indeed, El Mouedden and co-workers observed *in vivo* that gentamicin induced in proximal tubules a marked apoptotic reaction wich (1) was detectable after 4 days of treatment and was most conspicuous after 10 days, (2) was dose-dependent, (3) occurred in the absence of necrosis and (4) was nonlinearily correlated with proliferative response (tubular and peritubular cells). Comparative studies revealed a parallelism among extends of phospholipidosis, apoptosis and proliferative response for three of the aminoglycosides used (gentamicin >> amikacin ≅ isepamicin). By contrast, they observe that netilmicin induced a marked phospholipidosis but a moderate apoptotis and proliferative response (El Mouedden *et al.*, 2000a). Their observations have been reproduced *in vitro* on cellular model using renal cell lines (LLC-PK1 and MDCK

cells) and non-renal cell line (embryonic fibroblasts) (El Mouedden *et al.*, 2000b) where apoptosis developed almost linearily, with time (1 to 4 days) and drug concentration (0.5 to 3mM). The addition of pan-caspases inhibitor, z-VAD-fmk or the overexpression Bcl-2 have been shown to prevent apoptosis arguing for a caspase-and mitochondria-dependent pathway leading to death by apoptosis.

Based on all the new elements that could explain the nephrotoxicity of aminoglycosides, **our objective was the identification of the molecular pathway leading to apoptotic cell death induced by gentamicin.**

In the beginning of our study, we also knew that gentamicin (the more apoptogenic and nephrotoxic compounds used by El Mouedden)(El Mouedden *et al.*, 2000b) accumulates mainly in the S1 and S2 part of proximal tubules in the kidney (Molitoris *et al.*, 1993; Wedeen *et al.*, 1983; Silverblatt and Kuehn, 1979) and that it enters proximal tubular cells by a processus that should be receptor-mediated through megalin binding (Moestrup *et al.*, 1995) and probably associated to binding of aminoglycosides to anionic phospholipids (Sastrasinh *et al.*, 1982; Schacht, 1979). After its up-take, gentamicin resides mainly inside the lysosomes, where it is entrapped due to the acidic environment that is responsible for its protonation. Inside these organites, gentamicin induces some lysosomal alterations such as phospholipidosis (see figure 19 for resume).

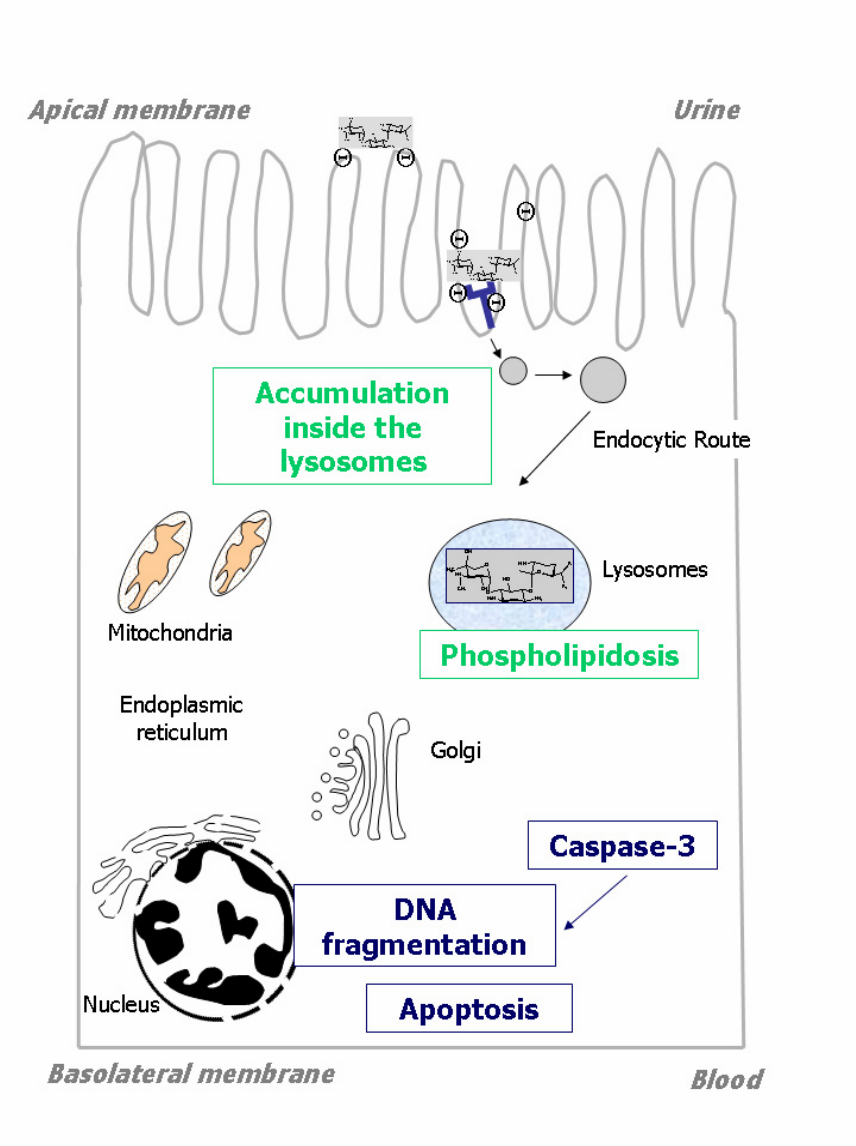


***Figure 19**: Schematic representations of important elements that have lead to our study*

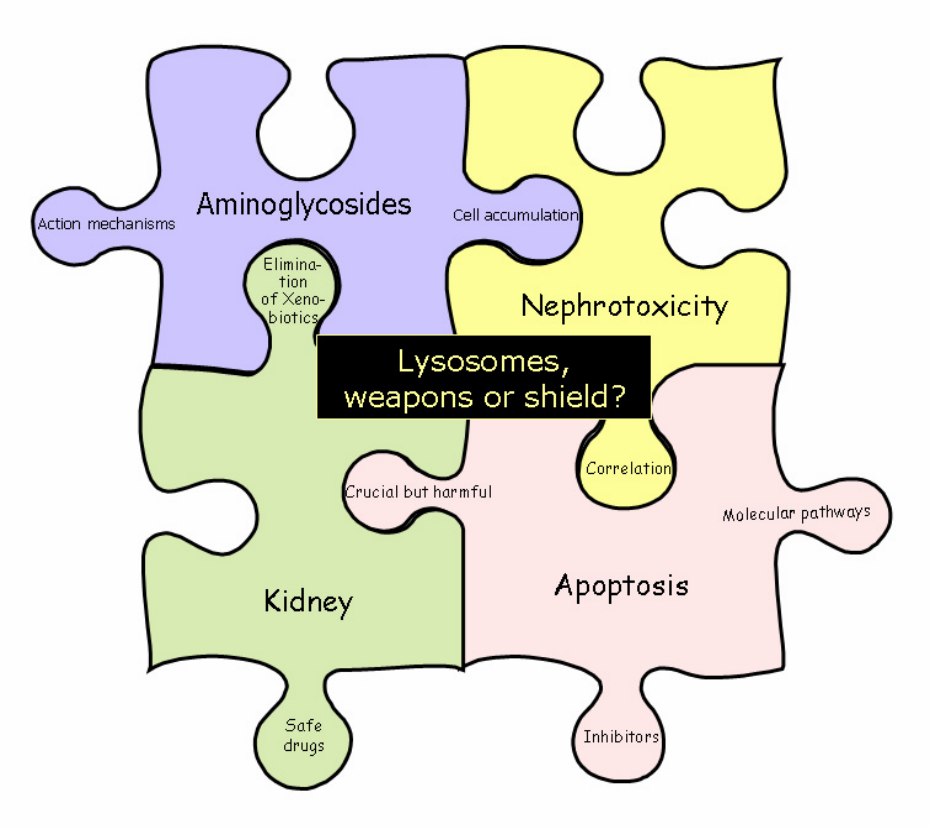
In addition, when we have started our studies, apoptosis was shown to be induced in two principal ways: (1) the receptor-mediated pathways and (2) the mitochondrial pathways, both of which converging in the final activation of specialized enzymes: the caspases. In the late1990s, researchers began to suspect the involvement of other intracellular organites in the apoptotic cascades and in particular the lysosomes. The involvement of lysosomes has been first described by Brunk and co-worker in 1997, where they have shown that cultured human fibroblasts subjected to photo-oxidation presented lysosomal membrane destabilization (Brunk *et al.*, 1997) and further apoptosis.

Later, the involvement of the lysosomes by their destabilization was confirmed for different death inducers such as MSDH (a lysosomotropic detergent, (Li *et al.*, 2000)) or by cholesterol oxidation products (Yuan *et al.*, 2000). Based on these observations, it has been further demonstrated that leakage of the lysosomal constituents to the cytoplasm can lead to apoptosis. As gentamicin is primarily accumulated inside these organites, the first part of our work was aimed to question if (1) lysosmes are destabilized in presence of gentamicin and if it is the case, (2) if this event occurred before mitochondrial activation, caspases activation and DNA fragmentation.

On the second part of our work we have analyzed if cathepsins are involved in gentamicin-induced apoptosis and in particular cathepsins B and D, which are the most relevant in this context. This has been tested by the addition of respective inhibitor of each of these cathepsins and by the evaluation of the percentage of apoptotic nuclei observed in these conditions. Finally, we have measured if cathepsin D is released from destabilized lysosomes by fractionation studies and the measure of cathepsin D activity in each fraction (nuclear, granular and supernatant fractions).

In the last part of this work, we have tested if gentamicin can induce apoptosis once having access to the cytosolic compartment as it could be the case after its release from permeabilized lysosomes. To evaluate this possibility, we have taken advantages of the electroporation technique, which allow the delivery of normally impermeant compounds into the cytosol of cell. Gentamicin could indeed activate apoptotic pathway(s) outside the lysosomes by interacting directly or indirectly with stress sensors, pro-apoptotic proteins or mitochondria.

Chapitre III: Results: Lysosomes, weapons or shield in gentamicin-induced apoptosis.



1. GENTAMICIN-INDUCED APOPTOSIS IN LLC-PK1 CELLS: INVOLVEMENT OF LYSOSOMES AND MITOCHONDRIA

Based on the previous observations (1) made by El Mouedden showing that gentamicin is able to induce apoptosis in conditions mimicking clinical situation on renal cell line, LLC-PK1 probably by activation of the mitochondrial pathway and the caspases and based on other observations (2) that gentamicin is localized mainly inside the lysosomes; our first objective was to analyze (1) if lysosomes are involved in gentamicin-induced apoptosis in LLC-PK1 cells by assessing lysosomal stability, (2) if mitochondria are activated by measuring the mitochondrial potential, the cytochrome c release and the activation of caspase-9. Finally, all these events were replaced from a sequential point of view in relation to caspase-3 activation and DNA fragmentation. Figure 20 illustrates the questions analyzed in the first part of this work and being elements of our first paper (Servais *et al.*, 2005). As gentamicin seemed to be able to destabilize lysosomes, we have tested this possibility on biophysical model (liposomes mimicking lysosomal membrane and others mimicking the inner and outer mitochondrial membrane).

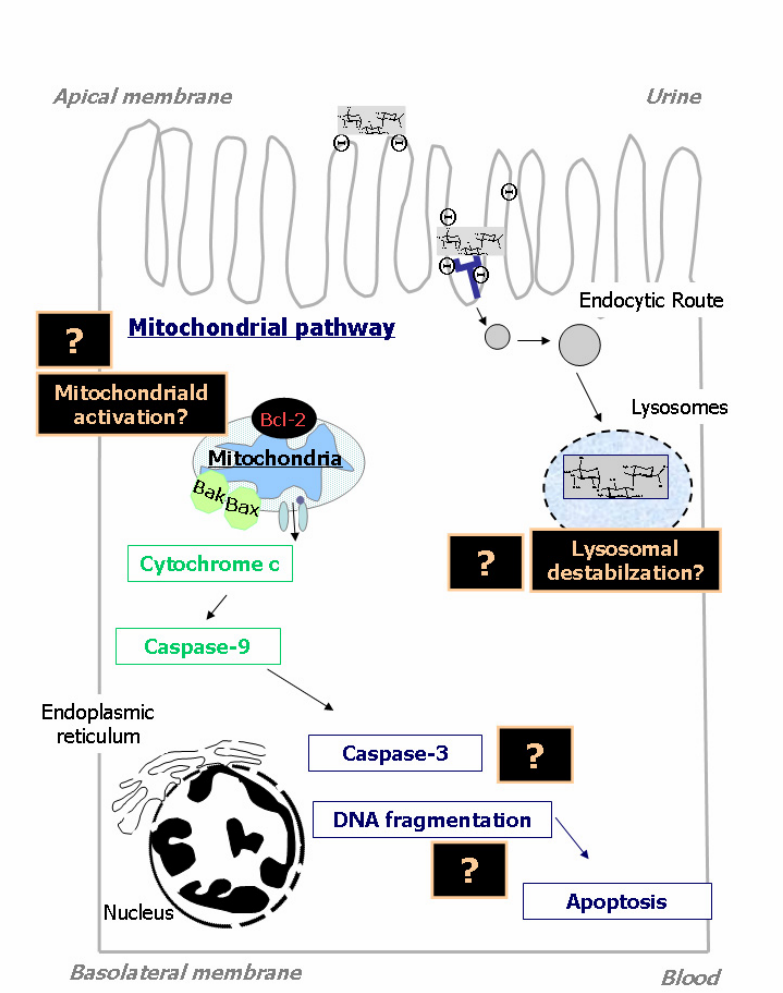


Figure 20: Schematic representations of questions raised in the fisrt part of our work.

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Gentamicin-induced apoptosis in LLC-PK1 cells: Involvement of lysosomes and mitochondria

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Abstract

Gentamicin accumulates in lysosomes and induces apoptosis in kidney proximal tubules and renal cell lines. Using LLC-PK1 cells, we have examined the concentration- and time-dependency of the effects exerted by gentamicin (1–3 mM; 0–3 days) on (i) lysosomal stability; (ii) activation of mitochondrial pathway; (iii) occurrence of apoptosis (concentrations larger than 3 mM caused extensive necrosis as assessed by the measurement of lactate dehydrogenase release). Within 2 h, gentamicin induced a partial relocalization [from lysosomes to cytosol] of the weak organic base acridine orange. We thereafter observed (a) a loss of mitochondrial membrane potential (as from 10 h, based on spectrophotometric and confocal microscopy using JC1 probe) and (b) the release of cytochrome *c* from granules to cytosol, and the activation of caspase-9 (as from 12 h; evidenced by Western blot analysis). Increase in caspase-3 activity (assayed with Ac-DEVD-AFC in the presence of z-VAD-fmk]) and appearance of fragmented nuclei (DAPI staining) was then detected as from 16 to 24 h together with nuclear fragmentation. Gentamicin produces a fast (within 4 h) release of calcein from negatively-charged liposomes at pH 5.4, which was slowed down by raising the pH to 7.4, or when phosphatidylinositol was replaced by cardiolipin (to mimic the inner mitochondrial membrane). The present data provide temporal evidence that gentamicin causes apoptosis in LLC-PK1 with successive alteration of the permeability of lysosomes, triggering of the mitochondrial pathway, and activation of caspase-3.

Keywords: Apoptosis; Lysosomes; Aminoglycosides; Gentamicin; Mitochondria

Introduction

Since its original description at a cellular and functional level (Kosek et al., 1974), gentamicin-induced nephrotoxicity has been the subject of extensive studies that have unambiguously identified proximal tubular cells as the almost exclusive and specific site for drug accumulation and early signs of ultrastructural and histological alterations (see Gilbert, 2000; Mingeot-Leclercq and Tulkens, 1999 for reviews). Lysosomes are the first and most conspicuous site of accumulation of gentamicin in proximal tubules after in vivo administration (Giurgea-Marion et al., 1986; Silverblatt and Kuehn, 1979) as well as in cultured cells incubated with this antibiotic (Tulkens and Trouet, 1978). The relationship between this subcellular localization of gentamicin and the onset of subsequent cell alterations and toxicity is, however, still unknown. In this context, two major advances have been made over the last years. First, drug localization studies using morphological approaches showed that, besides lysosomes, gentamicin has also partially access to the Golgi complex from where it traffics to the endoplasmic reticulum, the cytosol, and the intermembrane space of mitochondria

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(Sandoval and Molitoris, 2004; Sandoval et al., 1998; Sundin et al., 2001). Second, apoptosis has been shown to occur early on in proximal tubules of animals treated with gentamicin under clinically-relevant conditions with respect to both dosages and treatment duration (El Mouedden et al., 2000a). This effect of gentamicin has been reproduced with both renal (LLC-PK1 and MDCK) and non-renal (fibroblasts) cell lines exposed to gentamicin, and was found to be directly related to the amount of drug accumulated by the cells (El Mouedden et al., 2000b).

Apoptosis is a highly regulated process that can be triggered by both exogenous and endogenous factors which can activate intracellular components such as caspases, AIF, cytochrome *c*, leading to the final condensation and fragmentation of the nucleus. Among the various subcellular organelles potentially involved in apoptosis, both lysosomes and mitochondria have been shown to send death signals through the activation of specific stress sensors (Ferri and Kroemer, 2001). For lysosomes, membrane rupture and release of acid hydrolases (as seen during oxidative stress or occurring through the action of oxidized lipids, lysosomal detergents, serum withdrawal, or Fas-ligation (Brunk et al., 1997; Guicciardi et al., 2000; Li et al., 2000; Neuzil, 2002; Yuan et al., 2002) appear critical. "Lysosomal pathway to apoptosis" is now an accepted terminology to describe this mechanism which seems to have been primarily identified in pathological situations (Guicciardi et al., 2004). For mitochondria, two main events leading to apoptosis are the disruption of the transmembrane potential (Lemasters et al., 1998) and the release of cytochrome *c* (Kluck et al., 1997; Zamzami et al., 1995), which contribute to formation of the so-called apoptosome leading to the successive activation of caspase-9 and of the excecutioner caspase-3.

In the present study, we have examined if gentamicin may alter lysosomal stability and trigger the mitochondrial pathway (loss of mitochondrial potential, release of cytochrome *c*, activation of caspase-9) in relation to the further activation of caspase-3 and the chromatin condensation. We have attempted to rationalize our observations by examining directly the effect exerted by gentamicin on model bilayers meant to represent the lysosomal and mitochondrial membranes when incubated at acid and neutral pH.

Methods

Materials. Dulbecco's Modification of Eagle's minimum essential Medium (DMEM) and Trypsin-EDTA were purchased from Life Technologies, Paisley, UK. Gentamicin sulfate (obtained as GEOMYCINE^{R}, the branded product registered for clinical usage in Belgium; mixture of C_1, C_{1a} and C_2 components) was kindly provided by Glaxo-SmithKline (Genval, Belgium) on behalf of Shering-Plough (Brussels, Belgium). Ac-DEVD-AFC, leupeptin, pepstatin, aprotinin, CHAPS, PMSF, DTT, oxidized glutathione, acridine orange, m-CCCP, sphingomyelin, cholesterol, calcein, Tris maleate, Tris base, NADH, pyruvate, bovine serum albumin (BSA) and cardiolipin were purchased from Sigma Chemical Co. (St. Louis, MO). Egg phosphatidylcholine and phosphatidylinositol (Grade 1) were purchased from Lipid Products (Nr Redhill, UK). In situ cell death detection kitR (TUNEL assay), DAPI and protein-A-agarose came from Boehringer-Mannheim (Mannheim, Germany; presently Roche Diagnostics, Basel, Switzerland). JC-1 came from Molecular Probes Inc. (Eugene, OR). z-VAD-fmk was purchased from Promega U.S. (Madison, WI). Laemmli buffer and Tween 20 were purchased from Bio-rad Laboratories (Hercules, CA), polyclonal rabbit IgG raised against a residues 287–306 of recombinant human anti-caspase-9 (cross-reacting with pig) came from Stressgen Biotechnologies Corp (Victoria, BC), polyclonal rabbit raised against residues 1–104 of horse cytochrome *c* from Santa Cruz Biotechnologies Inc (Santa Cruz, CA), peroxidase-labeled animal anti-rabbit IgG from BD Transduction Laboratories (San Diego, CA), the nitrocellulose membranes (Hybond C) from Amersham Biosciences, UK, Ltd. (Little Chalfont, Bucks., England) and the SuperSignalR West Pico Chemiluminescence Substrate from Pierce (Rockford, IL).

Cells and treatments. All experiments were performed with LLC-PK1 (Lilly Laboratories Culture-Pig Kidney Type 1) cells as obtained from American Tissue Culture Collection (LGC Promochem, Teddington, UK) as ATCC CL-101. Cultures were grown in Dubelcco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum in an atmosphere of 95% air −5% CO_2. Cells were subcultured twice a week and used when reaching approximately 80% of confluence. All gentamicin solutions were adjusted to pH 7.4 prior being added to the culture medium.

Detection and enumeration of apoptotic cells. Cells were detached by trypsinization in Ca^{++}/Mg^{++}-free Hank's medium, pelleted by centrifugation at 1200 rpm for 10 min, washed three times with gentle resuspension and repelleting in ice-cold Phosphate Buffer Saline (PBS). Staining of DNA to reveal apoptotic bodies was made with 4′,6′-diamidine-2′-phenylindole (DAPI). Cells were resuspended in PBS, fixed in 4% paraformaldehyde for 30 min, spread on polylysine-coated slides, allowed to dry out for a few hours and incubated with the stain (1 μg/ml in methanol) for 30 min at 37 °C. Samples were then mounted in Mowiol/1, 4-diazabicyclo[2.2.2]octane (DABCO). Enumeration of apoptotic nuclei was made on slides picked up at random, using a Zeiss light microscope with 63 × oil-immersion objective, and counting about 500 cells. Clusters of apoptotic bodies were given as a single count. Data were expressed as the percentage of apoptotic nuclei relative to total number of nuclei counted.

Detection of necrotic cells. Necrosis was analyzed by following the release of lactate dehydrogenase (LDH) from cells into the medium after incubation with gentamicin as described earlier (Montenez et al., 1999). Results were expressed as the percentage of activity detected in the media over the sum of the activities in the media and in cells.

Measurement of caspase-3 activity. This was determined on the cell lysates (typically 50 µg protein per assay, as measured by a modified Bradford's method (Ramagli, 1999), using *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC) (Thornberry et al., 1997) as previously described (El Mouedden et al., 2000b) except that the lysis buffer was made of 10 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid], 2 mM EDTA [ethylene diamine tetraacetic acid], 0.1% CHAPS [3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulphonate], 5 mM DTT [1,4-Dithiotreitol], 1 mM PMSF [phenylmethylsulfonyl fluoride], 10 µg/ml pepstatin A, 10 µg/ml aprotinin, 20 µg/ml leupeptin and 1 mM oxidized glutathione. Kinetic fluorometric measurement of the release of 7-amino-4-trifluoromethyl coumarin was made continuously over 120 min to check for assay linearity. Specificity for caspases was assessed by running parallel samples in the presence of the pan-inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk). In preliminary experiments, samples of lysates of control and gentamicin-treated cells were incubated with increasing concentrations of z-VAD-fmk. A plateau of inhibition was observed at about 100 µM, and this concentration was, therefore, used for all subsequent determinations. Because the extent of inhibition by z-VAD-fmk was low in control cells (about 30%), and did not exceed about 63% in gentamicin-treated cells (3 mM, 48 h), we checked to what extent the increase in activity, as detected with Ac-DEVD-AFC, was truly related to apoptosis. For this purpose, cells were exposed to campthothecin (10 mg/L) for 16 h, which induced marked apoptosis (about 50%) and a 30-fold increase in caspase activity (as measured with Ac-DEVD-AFC), which was inhibited to more 84% in the presence of z-VAD-fmk.

Assessment of lysosomal membrane integrity. The integrity of the lysosomal membrane and the maintenance of a lysosomal-cytosolic pH gradient was assessed using the acridine orange relocation technique (Zdolsek et al., 1990). In this approach, cells are incubated with a fluorogenic organic weak base which diffuses into cells and accumulates in lysosomes and related acidic vacuoles by proton-trapping (Olsson et al., 1989). This accumulation produces a change in the fluorescence emission of the probe (from green to red for acridine orange, due to concentration-dependent stacking of the molecules). Disruption of the lysosomal membrane and/or marked change in lysosome pH can, therefore, be assessed by measuring the change in emission ratio in comparison to controls and by visual inspection of the cells by confocal microscopy (Brunk and Svensson, 1999; Li et al., 2000; Olsson et al., 1989; Zdolsek et al., 1990). For our experiments and unless stated otherwise, cells were loaded with acridine orange (5 µg/ml) in complete growth medium for 15 min at 37 °C, rinsed with DMEM and then incubated with gentamicin in Hanks′ Balanced Salt Solution (HBSS). *O*-methylserine dodecylamide hydrochloride (MSDH), a lysosomotropic detergent, was used as a positive control (Li et al., 2000). At appropriate times, cells were rinsed off from gentamicin or MSDH in ice-cold PBS supplemented in 3.6 mM $CaCl_2$ and 3 mM $MgSO_4$, and immediately observed using an Axiovert confocal microscope (Zeiss, Oberkochen, Germany) coupled to an MRC1024 confocal scanning equipment (Bio-Rad, Hemel, UK) operating at a λ_{exc} of 488 nm and at λ_{em} of 520 nm and 615 nm. For quantitative analysis, whole cells sheets were examined with a Fluorocount Microplate Fluorometer (Packard Instruments) with the excitation wavelength set of 485 nm and readings made at 530 nm and 620 nm. We checked that neither gentamicin nor MSDH interfered with the acridine orange fluorescence.

Disruption of mitochondria membranes potential. The mitochondrial potential ($\Delta\psi_m$) was measured using the fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolcarbocyanine iodide (JC-1), (Reers et al., 1991) which exhibits a potential-dependent accumulation in mitochondria. As for acridine orange, the fluorescence of JC-1 is influenced by its concentration with a green light (530 nm) being emitted in dilute solutions, and a red light (595 nm) when molecules aggregate at larger concentrations. This method has been validated for a reliable analysis of $\Delta\Psi_m$ changes in mitochondria (Salvioli et al., 1997). For our experiments, control and gentamicin-treated cells were incubated with JC-1 (6.7 µg/ml [10 µM]; extemporaneously prepared from a 1 g/L stock solution in DMSO) for 30 min at 37 °C in the dark. Cells were then rinsed with PBS supplemented in 3.6 mM $CaCl_2$ and 3 mM $MgSO_4$, and examined by confocal microscopy (as described above for the acridine orange studies) for qualitative studies or in a Fluorocount Microplate Fluorometer (Packard Instruments), with excitation wavelength set at 485 nm and readings made at 530 nm and 610 nm for quantitative studies. Carbonyl cyanide m-chlorophenylhydrazone (m-CCCP), a protonophore and uncoupler of oxidative phosphorylation in mitochondria (Laval and Little, 1977) was used as positive control at 20 µM for up to 10 h (Lim et al., 2001). We checked that neither gentamicin, nor mCCCP interfered with JC-1 fluorescence.

Preparation of a granules fraction and a cell supernatant. A granule fraction, containing the bulk of mitochondria and a cell supernatant were prepared by differential centrifugation from homogenates of LLC-PK1 using the general procedures set up for other cultured cells in our

laboratory (Tulkens et al., 1974b). In brief, cells collected by gentle scraping in 0.25 M sucrose −20 mM Tricine pH 7.4–1 mM EDTA pH 7.4 (Hayashi et al., 2000) and homogenized in an all-glass Dounce tissue grinder. The resulting homogenate was subjected to 3 successive low speed centrifugations (600 × *g* for 10 min) to obtain a fraction containing nuclei and unbroken cells, followed by a high-speed centrifugation (145,000 g for 30 min) to yield a granules fraction and a final supernatant. The content of each fraction in marker enzymes of the mitochondria (cytochrome *c* oxidase [E.C. 1.9.3.1]) and of the soluble proteins (lactate dehydrogenase [E.C. 1.1.1.27]) was measured using previously described techniques (Renard et al., 1987; Tulkens et al., 1974a).

Immunoprecipitation and detection of cytochrome c the cell granules fraction and supernatant. Samples were incubated overnight at 4 °C in presence of anti-cytochrome *c* antibody (Santa Cruz Biotechnology) in a volume of 1 mL, thereafter mixed with 30 µL Protein-A-agarose for 2 h at 4 °C, and centrifuged at 16,000 × *g* for 5 min at room temperature. The pellets were washed at 4 °C with PBS, 0.5 M NaCl, and twice with PBS. The final pellets were resuspended in Laemmli buffer, mechanically disrupted by forcing them 5 times through a 20-gauge needle, and subjected to separation by electrophoresis in 18% polyacrylamide gels prepared in 25 mM Tris-192 mM glycine buffer pH 8.3 and using the same solution supplemented with 0.1% SDS as running buffer. Proteins were transferred to 6 × 8 cm nitrocellulose membranes (Hybond C). The membranes were blocked for 1 h in Tris-buffered saline containing 5% fat-free milk proteins and 0.1% Tween 20 (TBS-T), and incubated overnight at 4 °C with polyclonal anti-cytochrome *c* antibodies (diluted in TBS-T containing 5% of BSA). Membranes were then washed four times (5 min each) in TBS-T, incubated with peroxidase-labeled anti-animal IgG antibody in that medium supplemented with 5% fat-free milk proteins, then visualized with SuperSignal West Pico Chemiluminescence Substrate.

Immunodetection of caspase-9 detection. Cells were collected as described for caspase-3 activity measurement and resuspended in Laemmli buffer. Detection of caspase-9 was performed by Western blot using the same general technique as for cytochrome *c*, using a rabbit anti-caspase-9 polyclonal antibody that, according to the manufacturer, detects both the pro-caspase-9 and the 36 kDa protein corresponding to the processed large subunit of caspase-9. Densitometric analysis of the gels was made by scanning them with a commercial scanner and analyzing the images with a public software (Image J v. 1.31, National Institutes of Health, Bethesda, MD; http://www.rsb.info.nig.gov/ij).

Studies with liposomes. Small unilamellar vesicles were prepared with compositions mimicking (i) the lysosomal and the outer mitochondrial membranes (cholesterol/phosphatidylcholine/phosphatidylinositol/sphingomyelin molar ratio 5.5:4:3:4), and (ii) the inner mitochondrial membrane (with cardiolipin replacing phosphatidylinositol while all other lipids remained in the same molar proportions). Vesicles were prepared by sonication in 40 mM Tris maleate buffer (345 mosm/kg) adjusted to pH 5.4 to mimick the lysosomal environment, and at pH 7.4 (338 mosm/kg) to mimick the mitochondrial environment, and in the presence of purified calcein (Van Bambeke et al., 1993; 23.11 mM for liposomes prepared at pH 5.4 and 19.55 mM for liposomes prepared at pH 7.4). The liposomes concentration was then adjusted to 50 µM (using the average molecular weight of the constituent lipids), exposed to gentamicin, mixed thoroughly for 20 s, and allowed to stand at 37 °C up to 16 h. Leakage of entrapped calcein was monitored by the increase in fluorescence of the samples, resulting from the dilution and release of self-quenching of this tracer (Weinstein et al., 1977). All fluorescence determinations were performed on an LS 30 fluorescence spectrophotometer (Perkin-Elmer Ltd., Beaconsfield, UK) using λ_{exc} and λ_{em} of 472 nm and 516 nm, respectively. The percentage of calcein released under the influence of drug was defined as $[(F_t - F_{contr}) / (F_{tot} - F_{contr})] \times 100$, where F_t is the fluorescence signal measured at time t in the presence of drug, F_{contr} is the fluorescence signal measured at the same time in the control liposomes, and F_{tot} is the total fluorescence signal obtained after complete disruption of liposomes by sonication (checked by quasi-elastic light spectroscopy).

Statistical analyses. The significance of the differences between paired values was tested by Student's *t* test, and that of two sets of data (kinetic studies) ANOVA analysis (Excel 97, Microsoft, Inc, Bellevue, WA). The significance of the differences between several sets of data was tested by ANCOVA analysis using XLSTATC version 6.0 (Addinsoft SARL, Paris, France). Correlations were calculated using GraphPad Prism version 2.1 for windows (GraphPad Software, San Diego, CA).

Results

Concentration-dependence of apoptosis and necrosis

In a first series of experiments, we examined the influence of increasing concentrations of gentamicin (1 to 12 mM) maintained for 24 h on apoptosis (DAPI staining) and cell necrosis (assessed by the release of LDH). Fig. 1 shows that the percentage of cells with evidence of apoptosis increased from about 2 to about 8% in the 0–3 mM range, with a more limited progression at larger concentrations (about 11% at 12 mM). In contrast, LDH release was not significantly different from controls up to a

gentamicin concentration of 3 mM, but markedly increased at larger concentrations.

Concentration- and time-dependence of apoptosis and activation of caspase-3

Having delineated the conditions in which necrosis became predominant (i.e., at a gentamicin concentration at 4 mM or above), we explored in more details the time- and concentration-dependence of the onset of gentamicin-induced apoptosis in the 0–3 mM range. We also measured the activity of caspase-3 (selected as one of the executioner caspases) in the same cells, Fig. 2 shows in combined fashion that both phenomena are time- and concentration-dependent. Considering the apoptosis process first (upper panel), a significant increase in the proportion of DAPI positive cells was already seen after exposing cells for 1 day at 1 mM of gentamicin, and this proportion increased steadily during the next 48 h. Larger concentrations of gentamicin (2 and 3 mM) accelerated the process and made it more intense with, however, a clear plateau after 48 h. Moving now to caspase-3 (lower panel), we see that the activity of caspase-3 showed a first, detectable but not statistically significant increase at 8 h before returning close to control values at 16 h. Thereafter, however, a marked activation was seen that proceeded, as for apoptosis, at a rate and an extent that were dependent on both gentamicin concentration and time of exposure. A maximal activity was obtained at 48 h for cells incubated with 1 and 2 mM gentamicin, and slightly earlier [30 h] for cells incubated with 3 mM gentamicin. Most conspicuously, a sharp decline was seen thereafter but the value reached at 72 h was still about twice that of the controls (this difference being statistically significant).

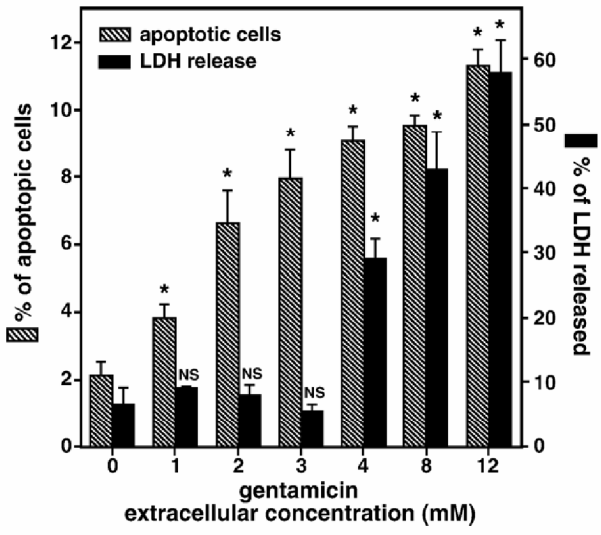

Fig. 1. Comparative increase in apoptosis index (DAPI staining) and in LDH release in LLC-PK1 cells exposed to increasing concentration of gentamicin (1 to 12 mM) for 24 h. Data are means ± SD of 3 independent determinations. NS and asterisks indicate the non-significant and significant changes, respectively, in treated cells compared to controls ($P < 0.05$; Student's paired tests).

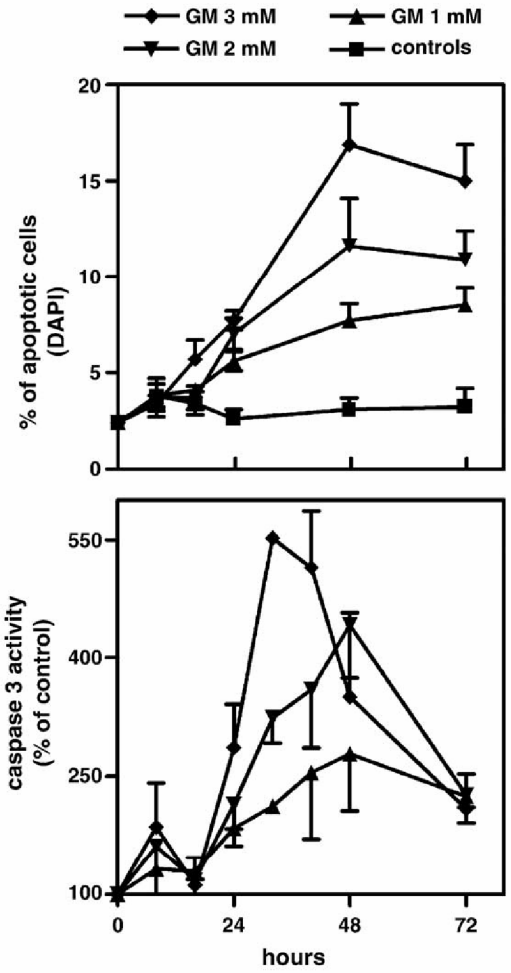

Fig. 2. Time-sequence of the development of apoptosis and activation of caspase 3 in LLC-PK1 cells exposed to increasing concentrations of gentamicin (GM; triangles, 1 mM; upside down triangles, 2 mM: diamonds, 3 mM). Values are given as arithmetic means ± standard deviation ($n = 3$; data points without bar have standard deviations smaller than the corresponding symbol size). Upper panel: detection of apoptosis by the DAPI staining (closed squares, untreated cells). Statistical analysis: the difference seen at 16 h between control cells and cells exposed to 3 mM gentamicin is significant ($P < 0.05$); all differences between control and treated cells are significant at 24 h or later ($P < 0.01$). Lower panel: change in the activity of caspase 3 in percent of the values observed in control cells (all values refer to z-VAD-fmk-inhibitable activities; mean activity in controls: 131 ± 19 μUnits/mg protein). Statistical analysis: (a) the changes seen between treated and control cells at 8 h are not significant; (b) from 24 h onwards, each set of values (corresponding to one treatment) is statistically significant from all others (corresponding to other treatments) and from controls by ANCOVA ($P < 0.05$).

Early lysosomal destabilization

Lysosomal destabilization was analyzed by the acridine orange relocation method using both spectrophotometric

analysis of whole cells and confocal microscopy. MSDH, a known lysosomotropic detergent was used as positive control (Li et al., 2000). As shown in Fig. 3, both gentamicin and MSDH produced an early (2 h) and steady (over the next 4 h) increase in the 530/620 nm ratio of the emitted light of acridine orange in whole cells. This increase was clearly concentration-dependent for gentamicin in the range investigated. Fig. 4 illustrates typical appearances and subcellular localizations of the tracer. In control cells (panels A and B), the tracer was almost exclusively found as red granules localized in the cytoplasm with a perinuclear distribution consistent with that of lysosomes, together with some faint green staining of the nucleoli. In cells treated with gentamicin for 3 h or more, red granules were still seen but many of them were surrounded by a yellow to green halo that seemed to smear into the cytoplasm. This feature, illustrated in panels C and D, was seen in significant proportion of cells incubated 3 h with 3 mM gentamicin. Other cells showed a more diffuse green staining in the cytoplasm together with a mixture of red and yellow granules (panel E; 4 h incubation with 2 mM gentamicin). These cells bore a striking similarity with those seen in large number upon treatment with MSDH (shown in panel F).

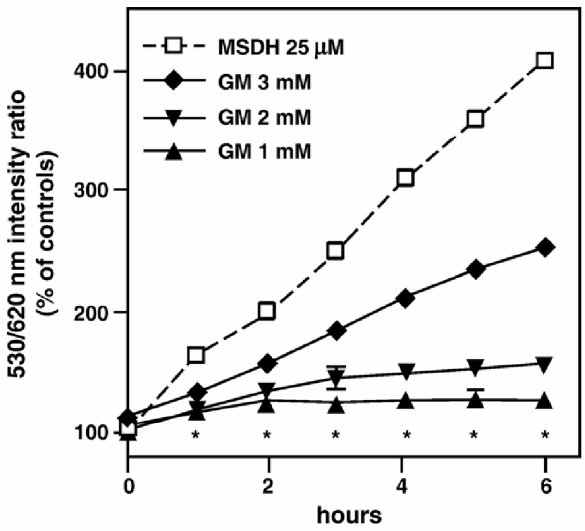

Fig. 3. Temporal variation of the 530 nm/620 nm fluorescence ratio of acridine orange in cells treated with gentamicin (GM; triangles, 1 mM; upside down triangles, 2 mM; diamonds, 3 mM) or MSDH (open squares; 25 µM) in percent of the values observed in controls. Cells were incubated with acridine orange (5 µg/ml) for 15 min and then returned to control medium (baseline) or to medium containing gentamicin or MSDH. Results are given as percentage of the values of controls (arithmetic means ± standard deviation; $n = 3$; when no SD bar is visible the standard deviation was smaller than the size of the symbol). A first analysis of all data points by ANOVA showed significant differences between the 4 groups ($P = 0.002$). Analysis by ANCOVA showed that each set of values (corresponding to one treatment) was significantly different from all others sets (corresponding to other treatments) or from controls ($P < 0.0004$). For cells treated with 3 mM GM, data points are significantly different from the corresponding controls as from 1 h ($P < 0.05$ by the Student's t test and for the other conditions from 2 h, denoted by asterisks at the bottom of the graph).

Decrease of mitochondrial transmembrane potential

The change of mitochondrial membrane potential ($\Delta\psi_m$) was monitored by measuring the shift in the fluorescence of the emitted light of JC-1, using, as for the acridine orange relocation studies, both spectrophotometric and confocal microscopy approaches. m-CCCP, a protonophore known to collapse the transmembrane mitochondrial potential (King et al., 1999; Laval and Little, 1977), was used as internal control. Fig. 5 shows the changes of the 610/530 nm ratio of the emitted light in whole cells as the percentage of that observed in control cells. As anticipated, the protonophore m-CCCP produced a fast and marked decrease of this ratio that remained low for several hours. In contrast, no significant effect of gentamicin was seen up to 8 h, after which a significant decrease (20–30%) was seen which persisted up to 24 h. Fig. 6 shows that JC-1 was distributed on a peripheral fashion in control cells appearing as elongated structures with an exclusively red color. In contrast, cells treated 12 h with 2 mM gentamicin showed a mixture of red and green structures. High magnification revealed that both structures were present in the same cells. In m-CCCP treated cells, most of the fluorescence was of green color, with a large part distributed in a diffused fashion in the cytosol, but also appearing associated with small, rounded shaped structures. Red structures were scarce.

Release of cytochrome c from mitochondria and activation of caspase-9

The release of cytochrome c in the cytosol resulting from activation of mitochondrial pathway was examined by Western blot analysis of granules and supernatant fractions prepared from control and cells exposed to gentamicin (2 mM) for 12 h. The granule fractions contained 90.4 ± 8.3 and 83.9 ± 4.7% of the activity of cytochrome oxidase, and 1.5 ± 1.3 and 2.0 ± 1.9% of the activity of the lactate dehydrogenase of the original homogenates obtained from control cells and cells incubated 12 h with gentamicin, respectively, whereas the supernatants contained 1.3 ± 1.7 and 2.5 ± 0.4% of the activity of cytochrome oxidase, and 96.6 ± 0.85 and 94.6 ± 1.87% of that of the lactate dehydrogenase ($n = 3$). Fig. 7 shows a typical image of a Western blot obtained with the granules fraction and the supernatant from control cells and cells treated with 2 mM gentamicin for 12 h. Cytochrome c was clearly detected in the supernatant in larger amounts than in control cells, whereas it was decreased in the granules fraction. In parallel, whole cell samples were examined by Western blot analysis for occurrence of processed, activated caspase-9. A distinct reaction product was seen in cells incubated for 12 and 16 h with 2 mM gentamicin at an apparent Mr of 36

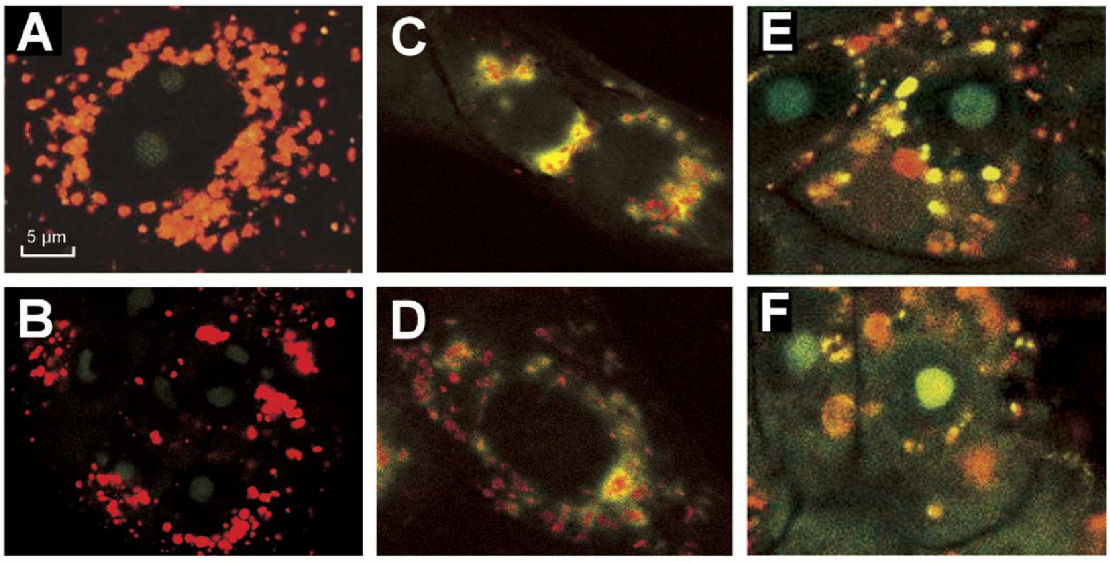

Fig. 4. Appearance of acridine orange-loaded LLC-PK1 cells in confocal microscopy. Cells were exposed to acridine orange (5 µg/ml) for 15 min and then returned to control medium for 3 h (A, B), or exposed to gentamicin (C and D, 3 mM, 3 h; E, 2 mM, 4 h) or MSDH (F, 25 µM, 3 h).

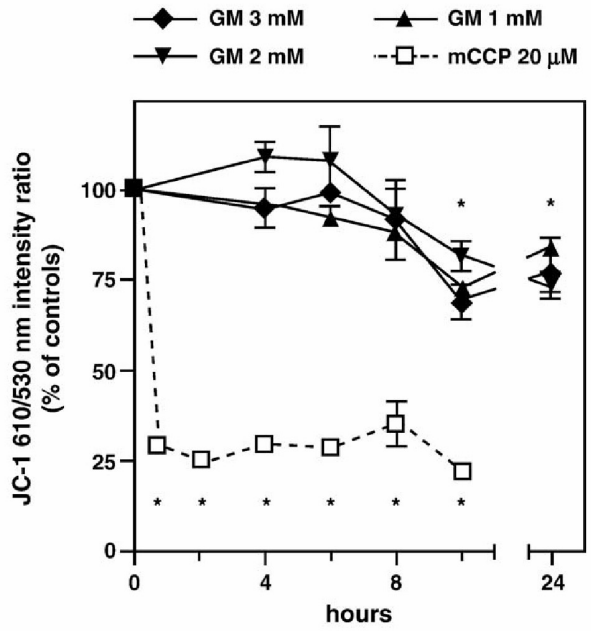

Fig. 5. Temporal variation of the 610 nm/530 nm fluorescence ratio of JC-1 in cells treated with gentamicin (GM 1 mM, triangles; GM 2 mM, upside down triangles; GM 3 mM, diamonds) or m-CCCP (open squares, 20 µM) in percent of the value observed in controls. Cells were incubated with gentamicin or m-CCCP for the times indicated in the abscissa and then exposed to JC-1 (6.7 µg/ml) for 30 min at 37 °C. Results are given as percentage of the values of control (arithmetic means ± standard deviation; $n = 3$; data points without bar have standard deviations smaller than the corresponding symbol size). All values from cells treated with m-CCCP are significantly different from control values. For gentamicin-treated cells, differences from control values are significant at 10 and 24 h ($P < 0.05$) and indicated by an asterisk on the graph to denote this point.

kDa (consistent with the known molecular weight of the large subunit of active caspase-9; densitometric analysis: 3.97 ± 0.01 [$n = 3$] increase over controls). This reaction product was not seen in cells incubated with gentamicin for 8 h only.

Changes in liposomes permeability

The permeabilizing potential of gentamicin towards lipid bilayers was examined on liposomes by measuring the release of entrapped calcein and the ensuing increase of its fluorescence due to its dequenching. Fig. 8 shows that gentamicin (2 mM) induced a fast (4 h) and marked increase in the fluorescence signal in liposome preparations when their composition mimicked that of the lysosomal membrane and the pH was adjusted to 5.4. Additional experiments were performed to explore the concentration-dependence of this release in the 0.001–3 mM range. Data could be fitted to a sigmoidal function ($R^2 = 0.9505$) with an EC_{50} of 22.5 µM (95% confidence interval, 6.7–75 µM). As shown in Fig. 8, the release of calcein proceeded at a slower pace when the pH was raised to 7.4. It was markedly delayed when examined at pH 7.4 with liposomes of a composition mimicking the inner membrane of mitochondria (i.e., in which phosphatidylinositol had been replaced by cardiolipin). In all cases, however, a similar extent of release (±60%) was obtained at 16 h.

Discussion

The data presented in this paper show that the onset of apoptosis induced by gentamicin on renal cell line is

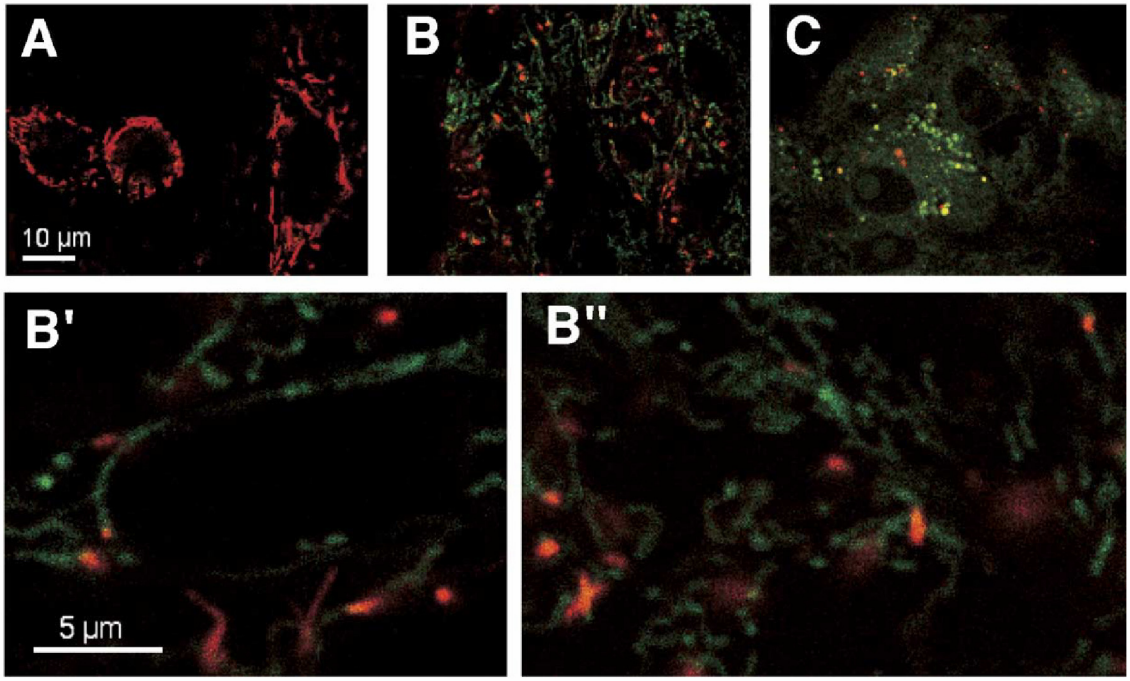

Fig. 6. Appearance of JC-1-loaded LLC-PK1 cells in confocal microscopy. A: control cells; B, B′, B″: cells incubated with gentamicin (2 mM, 12 h); C: cells incubated with m-CCCP (20 µM, 2 h). Cells were thereafter exposed to JC-1 (6.7 µg/ml) for 30 min at 37 °C.

preceded by lysosomal destabilization followed by definite signs of mitochondrial alterations known to be related to the activation of the mitochondrial pathway of apoptosis. This clearly appears from the examination of the data presented here, namely the successive (i) shift in acridine orange emission spectrum and its subcellular redistribution from lysosomes to cytosol), (ii) change in emission spectrum of the mitochondrial probe JC-1 associated with a change in subcellular localization of cytochrome *c*, and evidence of activation of capase-9, and (iii) fragmentation of nuclei and increase in activity of caspase-3. Apoptosis has been shown to develop at concentrations (0–3 mM) at which necrosis was still absent, demonstrating that it is a specific event most likely related to the triggering of the apoptotic machinery by gentamicin. While the present study does not provide a definitive model of pathogenic progression, it nevertheless provides a temporal sequence of alterations that can be used for further analysis of causal relationships.

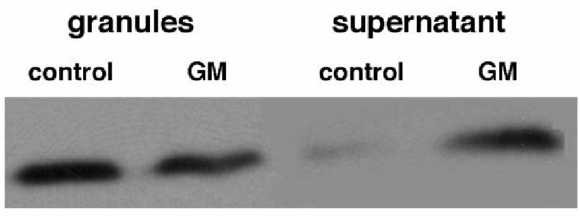

Fig. 7. Release of cytochrome *c* from mitochondria in cells exposed to gentamicin as demonstrated by Western blot of a granule fraction and a supernate prepared from control cells and cells incubated with 2 mM gentamicin for 12 h. Equivalent amounts of protein from each sample (158 µg) were mixed with 5 µL of polyclonal anti-cytochrome *c* IgG (1:200 dilution of a 200 µg protein/ml).

A key, initiating role of lysosomes in gentamicin-induced apoptosis in proximal tubular cells as well as in cultured cells was suspected (El Mouedden et al., 2000a, 2000b) based on the fact that this antibiotic most conspicuously accumulates in lysosomes both in vivo (Giurgea-Marion et al., 1986; Silverblatt and Kuehn, 1979) and in vitro (Sundin et al., 2001; Tulkens and Trouet, 1978). We now present direct evidence that lysosomes may suffer from early and significant destabilization of their membrane, as evidenced by our data with acridine orange. Acridine orange is a fluorescent dye that emits in green in diluted solutions but for which molecular stacking induces its emission to be shifted to red at high concentrations (Millot et al., 1997). As a weak organic base, acridine orange concentrates in the lysosomes of cultured cells (Olsson et al., 1989) explaining both the typical distribution and emission wavelength seen in control cells. A shift towards a green color, and a cytoplasmic staining are therefore highly indicative of either (i) a dissipation of the lysosome-cytosol pH gradient (suppressing the motive force that maintains the dye in lysosomes), or (ii) a loss of the integrity of the lysosomal membrane which will also result in the loss of transmembrane pH gradient. We have no simple way to distinguish between these two non-mutually-exclusive hypotheses from the present data. We may, nevertheless, probably dismiss the possibility of a simple and selective loss of lysosomal/cytosol pH gradient. Gentamicin, indeed, has been shown not to induce significant change of lysosomal pH in fibroblasts incubated for up

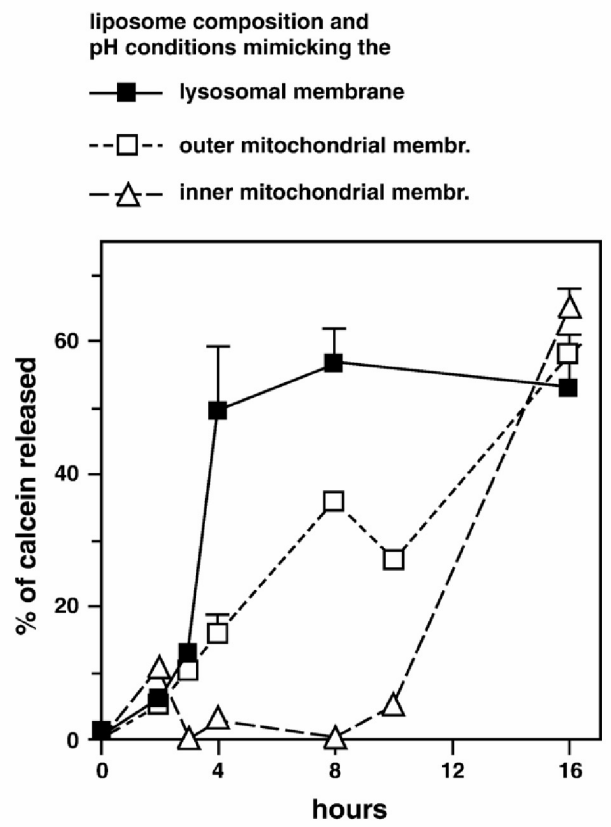

Fig. 8. Time-dependence of calcein release from liposomes (50 μM in average lipid) upon incubation at 37 °C with gentamicin (2 mM). The membrane compositions and pH were adjusted to mimic the lysosomal, outer mitochondrial and inner mitochondrial membranes, and their corresponding environments (closed squares, cholesterol/phosphatidylcholine/sphingomyelin/phosphatidylinositol [5.5:4:3:4 molar ratio], pH 5.4); open squares, same composition but pH 7.4; triangles, phosphatidylinositol was replaced by cardiolipin, pH 7.4). Results are given as the amount of calcein released in percentage of the maximal releasable amount (determined by complete disruption of the liposomes). Data are arithmetic means ± standard deviation (n = 3; when no SD bar is visible the standard deviation was smaller than the size of the symbol). A first analysis of all data points by ANOVA showed no significant differences between groups (P = 0.41). Analysis by ANCOVA, however, showed that each set of values (corresponding to one condition of liposome composition and/or of pH) was significantly different ($P < 0.001$) from all others or from controls (no gentamicin added) ($P < 0.001$). For each data point corresponding to liposomes of a composition mimicking that of the lysosomal or outer mitochondrial membrane, all values are significantly different from the corresponding controls as from 2 h of contact with gentamicin. For liposomes of a composition mimicking that of the inner mitochondrial membrane, significant differences are only observed at 2, 4, 10, and 16 h.

to 24 h in the presence of 2.2 mM of gentamicin, based on studies using the endocytizable pH probe FITC dextran (Oshima et al., 1986). In contrast, our results with MSDH, which induces a leakage of acridine orange out of lysosomes as in gentamicin-treated cells, support the concept of membrane disruption. MSDH, indeed, is a detergent that accumulates in lysosomes and produces bilayer disruption when exposed to acidic pH (Firestone et al., 1979; Li et al., 2000). Because lysosomes form a heterogenous population with respect to endocytosis and potential damage to membranes (Nilsson et al., 1997), and since gentamicin was present all along our experiments, the overall process may appear continuous when examined by spectrophotometry. The relocalization of acridine orange has already been extensively used to evidence the loss of lysosomal membrane integrity by L-Leucyl-L-Leucine methyl ester (Uchimoto et al., 1999). Besides MSDH, the role of lysosomal disruption as an initial event in triggering apoptosis has also been clearly observed with a variety of membrane-damaging compounds or molecules that accumulate in lysosomes or bind to lysosomal membrane, such as digitonin (Ishisaka et al., 1998), α-tocopheryl-succinate (Neuzil et al., 1999), 6-hydroxydopamine (Takai et al., 1998), synthetic retinoid (Zang et al., 2001), bile salts (Roberts et al., 1997), sphingosine (Kagedal et al., 2001). We also know that apoptosis can be selectively triggered by direct damage to lysosomes resulting from photooxidation (Brunk et al., 1997), or oxidative stress induced by naphthazarin (Roberg and Ollinger, 1998), or hydrogen peroxide (Brunk et al., 1995; Nilsson et al., 1997; Ogawa et al., 2004).

Whereas the potential role of lysosomes in initiating apoptosis is now widely accepted, there is no consensus as to whether lysosomal rupture can directly result in the activation of the executioner caspases or whether the lysosomal pathway needs to be relayed by the mitochondrial one (Guicciardi et al., 2004). In the present work, we demonstrate that lysosomes are destabilized after 2 h of incubation of the cells with gentamicin, and that the mitochondrial pathway leading to apoptosis is activated at 10 h. We do not know to what extent lysosomal changes are related to mitochondrial activation. But we cannot exclude either that lysosomal-mitochondrial cross-talk constitutes an amplifying loop in the apoptotic process. A loss of mitochondrial potential heralds indeed an alteration in the mitochondrial permeability properties and is known, since several years, to precede the onset of apoptosis (Lemasters et al., 1998). Recently, another group of investigators (Sandoval and Molitoris, 2004) using rhodamine B hexyl ester as probe also described a loss of mitochondrial potential in LLC-PK1 cells incubated with 1 mg/ml gentamicin (approximately 2.15 mM). A significant decrease was observed after 4 h and progressed over time to reach 20% of the control values at 8 h. In comparison, the modest effect exerted by gentamicin on the mitochondrial potential in our study may seem surprising at first glance. It could be related to the use of a different probe (and JC-1 used here may actually be one of the most faithful probes in this respect) (Salvioli et al., 1997). But, in a more general context, it could simply be related to the fact that gentamicin is a low apoptosis inducer the influence of which on mitochondria is expected to be weaker than that of stronger inducers. It is interesting to note that sphingosine, another weak apoptosis inducer, causes also only a modest decrease in mitochrondrial potential (Kagedal et al., 2001). Our morphological studies also showed that gentamicin differently affected

individual mitochondria within the same cell, suggesting that the globally modest decrease seen in the spectrophotometric analysis could actually cover major local changes.

The main point, however, is that the change in mitochondrial potential demonstrated here is accompanied by a partial redistribution of cytochrome *c* from the granules fraction to the cytosol and by the activation of caspase-9. Cytochrome *c* released from mitochondria is a well-known trigger of apoptosis because it can interact in the cytosol with the Apaf-1 protein and the inactive form of caspase-9 to form the apoptosome (Li et al., 1997). This leads to activation of caspase-9 (Kandasamy et al., 2003; Zou et al., 1997) and other caspases, including caspase-3. The mitochondrial pathway to apoptosis is known to be blocked by the overexpression of Bcl-2 (Wang et al., 2004). Our previous study demonstrated that gentamicin-induced apoptosis in LLC-PK1 cells is largely prevented in cells overexpressing Bcl-2 (El Mouedden et al., 2000b). Thus, one may probably exclude a direct effect on caspase-3 without mitochondrial relay in gentamicin-induced apoptosis.

We do know whether the triggering of the mitochondrial pathway is due to a direct effect of the antibiotic on mitochondria, or whether it results from some other mechanism. The first hypothesis implies that gentamicin has access to the mitochondria, which may simply result from its release from lysosomes as we suggest here. It is interesting to note, in this context, that gentamicin is capable of permeabilizing liposomes with a composition that mimics that of the outer mitochondrial membrane at pH 7.4. While this process is less rapid than at pH 5.4, it is nevertheless faster than what is observed for liposomes of a composition mimicking the inner leaflet of the mitochondrial membrane. The latter observation is consistent with data (Mather and Rottenberg, 2001) showing that aminoglycosides have a larger affinity for phosphatidylinositol (one of the acidic phospholipids present in lysosomal and outer mitochondrial membrane) than for cardiolipin (which is abundant in the inner mitochondrial membrane). It remains, however, to be seen whether gentamicin can also destabilizes the lysosomal membrane in cultured cells or in renal tissue, although the concentrations needed (EC_{50} of 22.5 µM at acid pH) are probably easily reached (see discussion in El Mouedden et al., 2000a, 2000b).

Possibly also, gentamicin could have a direct access to mitochondria without rupture of lysosomes thanks to its retrograde transport from the Golgi complex through the endoplasmic reticulum from where translocation of the drug to the cytosol was shown to occur (Sandoval and Molitoris, 2004). Retrograde transport of drugs, and participation of the Golgi structures and the endoplamic reticulum in their intracellular trafficking is indeed a rather common event which has been advocated in other cases of drug-induced toxicities such a those observed with the cationic amphiphiles (Ruben et al., 1991). Incidentally, one of the reasons for gentamicin to be a weak apoptosis inducer may actually be the fact that only a small part of the cell-associated drug eventually reaches mitochondria (probably less than 10%, as judged by the most recent morphological data obtained with LLC-PK1 (Sandoval and Molitoris, 2004).

Finally, other factors released from lysosomes than gentamicin it-self could also elicit a loss of mitochondrial potential. Lysosomal proteases, for instance, can activate Bid or other cytosolic pro-enzymes which would then induces the release of cytochrome *c* (Brunk et al., 2001; Guicciardi et al., 2000; Roberg et al., 1999; Stoka et al., 2001; Zhao et al., 2001), inducing thereby the formation of the apoptosome and the subsequent caspases activation. Formation and release of reactive oxygen species, which have been implicated in the development of apoptosis by gentamicin both in kidney glomeruli (Martinez-Salgado et al., 2004) and in the organ of Corti (Nagy et al., 2004), could also play a crucial role.

One may wonder why apoptosis seems to reach a plateau and does not proceed further after 1 to 2 days of culture with gentamicin. This plateau may actually be only apparent, since cells undergoing apoptosis are likely to quickly detach from the growing surface and will, therefore largely escape detection. The potential presence of cells truly refractory to gentamicin is, in any case, unlikely since for higher concentrations, apoptotic death shift to necrotic death as shown by LDH release measurement. A key-factor in determining the type of cell death (necrosis vs. apoptosis) mediated by the lysosomal pathway seems to be the magnitude of lysosomal permeabilization and, consequently, the amount of proteolytic enzymes released into the cytosol (Li et al., 2000). A complete breakdown of the organelle with release of high concentrations of lysosomal enzymes into the cytosol results in unregulated necrosis, whereas partial, selective permeabilization triggers apoptosis (Guicciardi et al., 2004).

In spite of these uncertainties, the present study opens potentially interesting perspectives for the unraveling of gentamicin-induced renal alterations. Although direct extrapolation from in vitro to in vivo data remains a risky exercise, LLC-PK1 cells have been widely used as a model for the study of various aspects of gentamicin-induced nephrotoxicity (Hori et al., 1984; Kohlhepp et al., 1994; Sandoval et al., 1998; Schwertz et al., 1986) and apoptosis (Shino et al., 2003; Yano et al., 2003). In the present study, large gentamicin concentrations (1–3 mM; approximately 0.5 to 1.4 g/L) have been used, which may raise questions about the significance of our results with respect to the situation prevailing in vivo (gentamicin typical serum concentrations are indeed in the 1–20 µM range). As explained earlier (El Mouedden et al., 2000b), these concentrations simply allow to obtain in LLC-PK1 cells cellular and intralysosomal drug concentrations of the same order of magnitude as those observed in proximal tubular cells of animals receiving clinically-relevant doses of gentamicin and in which apoptosis has been demonstrated (El Mouedden et al., 2000a; most interestingly, this study shows that increasing the dose administered to animals to supraclinical ones shifts the response to necrosis as observed

here). Since apoptosis is generally considered as a pathological event when developing in kidney after exposure to drugs (Lieberthal et al., 1996; Tanimoto et al., 1993; Thevenod, 2003), the present data may provide the so far missing link between gentamicin accumulation in lysosomes (Giurgea-Marion et al., 1986; Silverblatt and Kuehn, 1979) and the onset of the cascade of cellular events leading to functional and pathologic signs of gentamicin nephrotoxicity (Gilbert, 2000; Tulkens, 1986).

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2. INVOLVEMENTS OF CATHEPSINS B AND D IN GENTAMICIN-INDUCED APOPTOSIS?

2.1. Introduction

In the precedent chapter, we show that in LLC-PK1 exposed to gentamicin (1 to 3 mM), lysosomes are the first organelles to be found altered. Indeed, after 2 hours of contact with the drug, lysosomal membranes are destabilized has shown by the relocation of acridine orange. Mitochondrial changes have only been detected after 10 hours of incubation by the observation of a decrease of mitochondrial membrane potential, this was assessed by JC-1. This mitochondrial event is accompanied at 12 hours by the release of cytochrome c (detected by western blot analysis of the different fractions, nuclear, granules and supernatant) and the activation of caspase-9 (detected by western blot on whole lysates). The final activation of caspase-3 (assessed by specific substrate Ac-DEVD-AFC) and the DNA fragmentation (detected by fluorescence microscopy using DAPI as dye) were both only detected after 24 hours. All these events are resumed in figure 21.

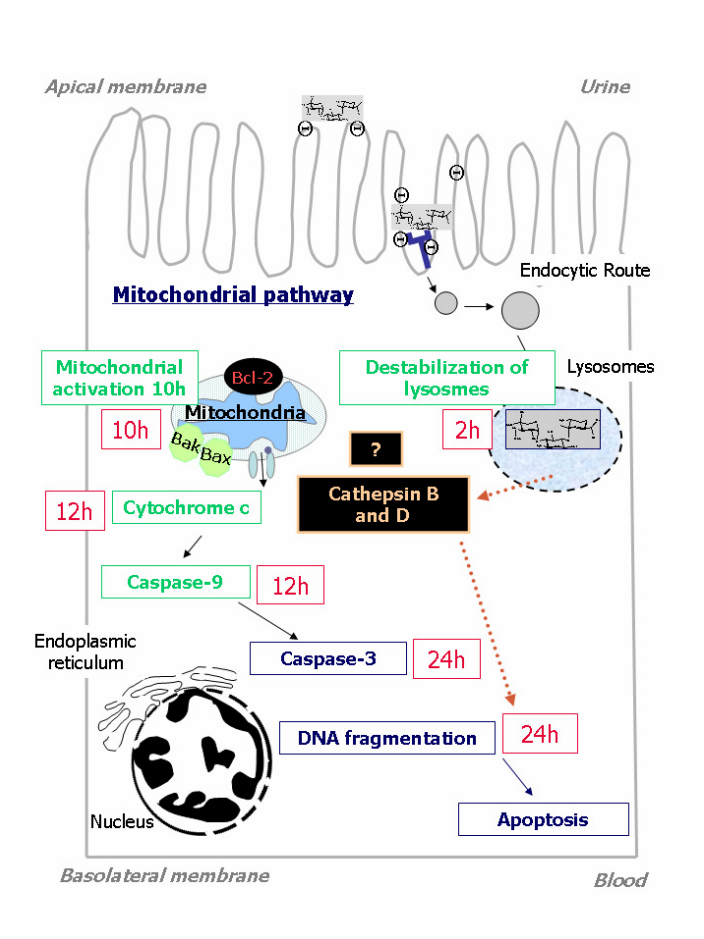

Figure 21: *Schematic representation of all apoptotic alterations observed in a proximal tubular cell exposed to gentamicin (1 to 3 mM)*

Thus in our observations, lysosomes seemed to be involved before the activation of the mitochondrial pathway. Between all the candidates that can link lysosomal to mitochondrial events, cathepsins have been shown to be important actors, and in particular cathepsins B and D. To assess their involvement in gentamicin-induced apoptosis in LLC-PK1 cells (1) we tested if their respective inhibitors, leupeptin and pepstatin A, can protect cells from apoptosis (apoptotic cells were quantified by fluorecence microscopy using DAPI dye as described in Servais 2005) (figures 22 and 23), and then (2) we have analyzed if cathepsin D can be released from the lysosomes to the cytosolic compartment to be able to induce apoptosis (this was assayed by fractionation and cathepsin D was assessed by the modified Barrett method) (figure 24).

2.2. Results

2.2.1. Protective effect of pepstatin A but not leupeptin

Leupeptin, a cystein proteases inhibitor is known to inhibit cathepsin B. LLC-PK1 cells were pre-incubated for 16 hours with leupeptin to ensure its capture by cells before the addition of gentamicin. After pre-incubation, fresh medium was added in presence or absence of gentamicin, alone or in combination with fresh solution of leupeptin. As no protection was observed by the addition of 25 or 50 µM of leupeptin in treated cells with gentamicin 2 mM for 32 hours and no differences were noted between control or treated cells with leupeptin 25 - 50 µM alone, we have used a higher concentration of leupeptin of 100 µM. The results are illustrated on figure 22 and show that leupeptin 100 µM is able to induce by it-self apoptosis in LLC-PK1 cells after 24 and 48 hours of incubation. The addition of gentamicin and leupeptin in LLC-PK1 cells demonstrate an "additive" apoptogenic effect.

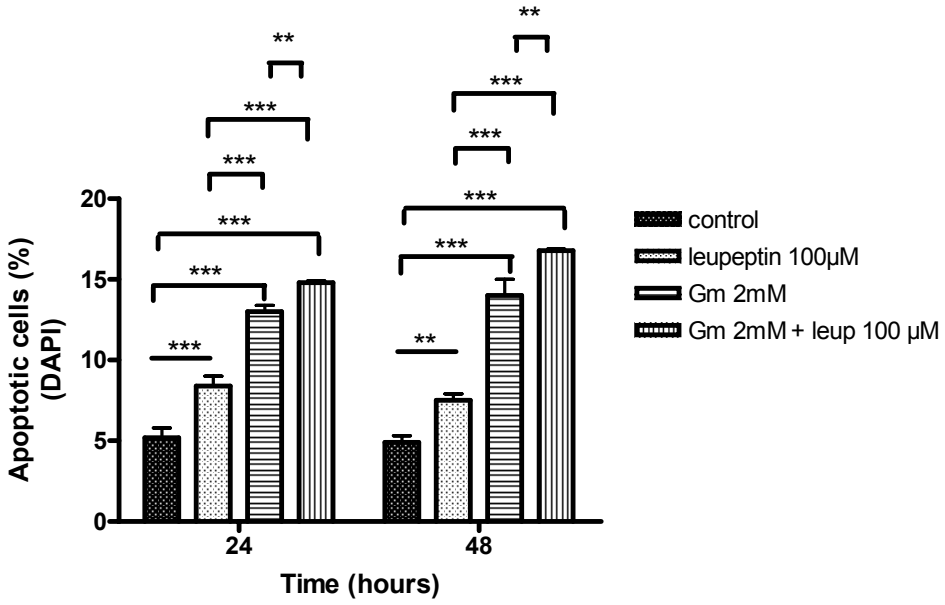

***Figure 22**: LLC-PK1 cells were pre-incubated for 16 hours with leupeptin A 100 µM, and freshly prepared medium containing or not gentamicin 2mM and /or leupeptin 100 µM were added for further incubation 24 or 48 hours. Apoptotic cells were determined by fluorescence microscopy using DAPI dye. Values are means ± standard deviations (n=3 in each group). Statistical analysis was performed using ANOVA test (Tukey-Kramer multiple comparisons test).*

LLC-PK1 cells were also incubated with another inhibitor, pepstatin A known to inhibit cathepsin D (but also pepsin and renin) and used in the same time-frame than leupeptin (pre-incubation of 16 hours follow by incubation with fresh solutions). Different concentrations of pepstatin (50-100-150) were analyzed and no protection was observed with concentration of 50 µM. The addition of 100 and 150 µM of pepstatin A demonstrated protection from gentamicin-induced apoptosis so we have used further 100 µM of pepstatin A. Results obtained are illustrated in figure 23 and show that pepstatin A 100 µM induces by it-self a very low increase of apoptosis after 24 hours which was not observed after 48 hours of incubation. The addition of this inhibitor in LLC-PK1 cells treated with gentamicin results in the reduction of the number of apoptotic cells and this was observed after 24 hours of incubation but also after 48 hours. However, this reduction can not reach control values.

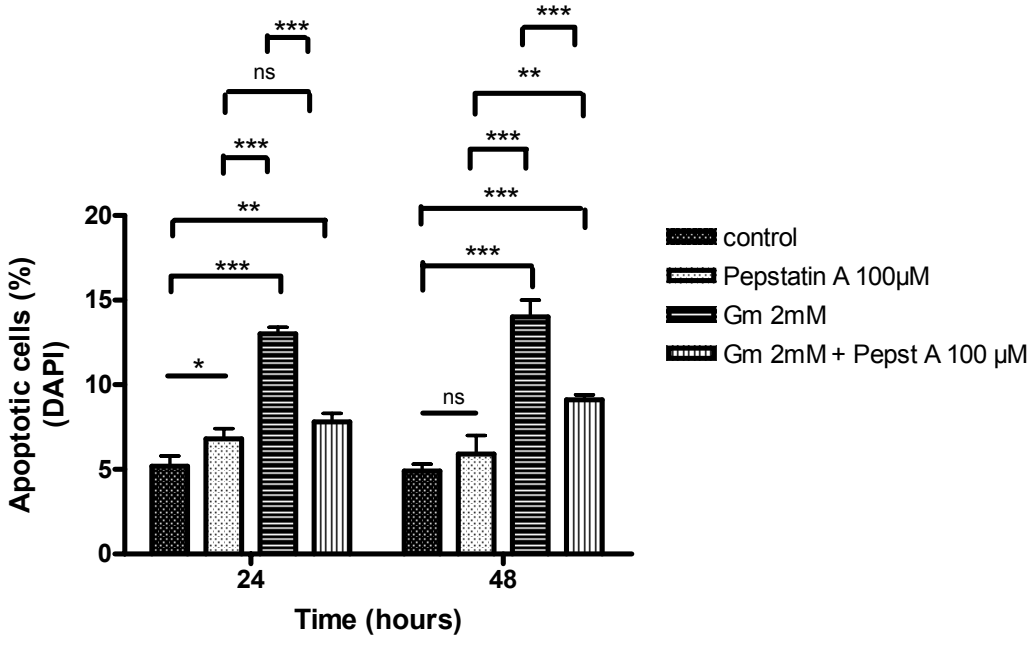

***Figure 23**: LLC-PK1 cells were pre-incubated with pepstatin A 100 µM for 16 hours, and freshly prepared medium containing or not gentamicin 2mM and/or pepstatin A 100 µM were added for further incubation (24 or 48 hours). Apoptotic cells were determined by fluorescence microscopy using DAPI dye. Values are means ± standard deviations (n=3 in each group). Statistical analysis was performed using ANOVA test (Tukey-Kramer multiple comparisons test).*

2.2.2. **Cathepsin D was not found released from the lysosomes to the cytosolic compartment**

In conclusion of these observations, cathepsin D could play a role in the apoptotic cascade induced by gentamicin but cathepsin B seems not to be involved. Thus, as relocation of cathepsins from the lysosomes to the cytosolic compartment is usually associated with lysosomal permeabilization, we checked that if cathepsin D can be released in gentamicin-induced apoptosis. This has been assessed by fractionation of LLC-PK1 cells treated or not with gentamicin (2 mM) and by measuring the cathepsin D activity (see additional materials) in each fraction (nuclear, granular and supernatant, fractions obtained as described in Servais (Servais *et al.*, 2005). Surprisingly, we were not able to detect any release of cathepsin D since the distribution of cathepsin D activity is similar between control and treated cells with gentamicin 2 mM cells. This was observed after 4 hours but also after 12 hours of incubation (figure 24) whereas we have previously described a destabilization of lysosomes significant from 2 hours of contact with the drug (Servais *et al.*, 2005). Our results were validated by the use of known lysosomal destabilizing agent the MSDH (*O*-methyl-serine dodecylamine hydrochloride) which allow us to detect a different redistribution of cathepsin D but these results should be confirmed.

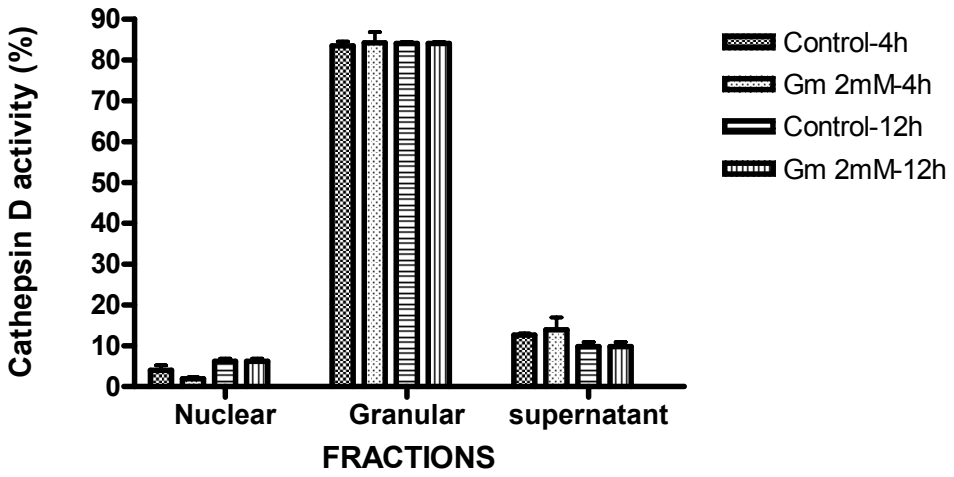

Figure 24: *Cathepsin D activity measured as using hemoglobbin as substrate in the different fraction of LLC-PK1 cells untreated or treated with 2mM of gentamicin. Values are means ± standard deviations (n=3 in each group)..*

2.3. Additional materials

The number of apoptotic cells was quantified by fluorescence microscopy using DAPI dye as described previously (Servais *et al.*, 2005) and fractionation was performed according techniques described previously (Servais *et al.*, 2005; Tulkens *et al.*, 1974).

Cathespin D activity:

Cathepsin D activity was determined as described by Barrett (Barrett A.J. and Kirschke H., 1977) by using haemoglobin as substrate of cathepsin D with some minor modifications. Briefly, lysates from each fraction (nuclear, granular and supernatant fractions) were mixed with TRITON X-100 2% (0.2% final concentration), with sodium formate buffer 1M pH 3.5 (final concentration 0.277 M) and with substrate haemoglobin 4% (final concentration 1.1%) and then incubated for 3 hours at 45°C. The reaction was terminated by adding 5ml of 3% trichloracetic acid on ice and precipitates were filtered at 4°C. The amount of peptides released was estimated by the Lowry method with minor modifications. Filtrates were mixed with Na_2CO_3 2% (final concentration:1.6%)

NaOH 3.75 N (final concentration 0.14N), sodium/potassium Tartrate 2% (final concentration 0.016%) and with $CuSO_4.5H_20$ 1% (final concentration 0.00041%) and incubated for 15 minutes at room temperature. Folin-Ciocalteu solution of 2N (0.083N) was added, mixtures were then incubated for 30 minutes and absorbance was then measured at 660nm. Cathepsin D activity was expressed as the percentage of activity found in each fraction in respect to the total activity.

2.4. Conclusion:

In conclusions of these additional results, it seems that cathespin B is not involved in gentamicin-induced apoptosis in LLC-PK1 cells whereas cathepsin D could be involved. However, surprisingly, no redistribution of cathepsin D activity has been observed in the different fractions in cells treated with gentamicin 2 mM in comparison to control cells whereas we were able to detect such changes with positive control, cells treated with MSDH. Thus, at this stage, we cannot involve or exclude cathepsin D in the apoptotic pathways activated by gentamicin in LLC-PK1 cells.

2.5. Discussion:

Between all the cathepsins, cathepsin B and D seemed to be the major ones involved in apoptotic pathways even if their real apoptotic target(s) are not yet defined unambiguously. Indeed, cathespin B has been shown to be involved in bile-acid induced hepatocytes apoptosis (Roberts *et al.*, 1997; Jones *et al.*, 1998; Faubion *et al.*, 1999; Canbay *et al.*, 2003), in TNF-α induced apoptosis of primary hepatocytes and tumor cells (Guicciardi *et al.*, 2000; Guicciardi *et al.*, 2001; Foghsgaard *et al.*, 2001) but also after brain ischemia (Yamashima *et al.*, 1998; Tsuchiya *et al.*, 1999). In our experiments, we found that inhibition of cathepsin B by the use of leupeptin (an inhibitor of serine and cystein proteases that has been shown to inhibit plasmin, trypsin, papain and cathepsin B (Aoyagi *et al.*, 1969) did not protect LLC-PK1 renal cells from gentamicin-induced apoptosis but interestingly enough, we found that leupeptin it-self is able to

induce apoptosis in these cells. The apoptogenic effect of leupeptin has been previously observed by Maeda and colleagues: indeed, they observed that incubation of primary culture of rat hepatocytes with leupeptin induce apoptosis (Maeda *et al.*, 1996). But how can we explain this pro-apoptotic ability? One possible explanation could be an apoptogenic effect resulting from the proteasome inhibition, indeed, a leupeptin analogue, Z-Leu-Leu-Leucinal has been shown to induce apoptosis in MOLT-4 cells and in L5178 cells by inhibition of the proteasome (Shinohara *et al.*, 1996) but more interestingly, it has also been shown that leupeptin inhibits directly trypsin-like proteasomal activity with concentration of about 50µM (Savory and Rivett, 1993).

If leupeptin could not afford protection against gentamicin-induced apoptosis, pepstatin A (a potent inhibitor of acid proteases including pepsin, renin and cathepsin D) seemed to protect LLC-PK1 cells from apoptosis suggesting a possible role of cathespin D in apoptotic cascade induced by gentamicin. Indeed, this cathepsin has been shown to be involved in different model of apoptosis including apoptosis induced by staurosporin (Bidere *et al.*, 2003; Johansson *et al.*, 2003), interferon-γ, Fas/CD95/APO-1 and TNF-α (Deiss *et al.*, 1996; Demoz *et al.*, 2002), oxidative stress (Roberg and Ollinger, 1998; Roberg *et al.*, 1999; Ollinger, 2000; Kagedal *et al.*, 2001a), sphingosine (Kagedal *et al.*, 2001b) and p53 (Wu *et al.*, 1998). To further characterize its involvement in gentamicin-induced apoptosis in renal cell line, we tried to analyse by fractionation studies if cathespin D can be released from lysosomes to the cytosolic compartment where it has been shown to induce apoptosis (Roberg *et al.*, 2002). Surprisingly, we were not able to find any relocation of cathepsin D after 4 and 12 hours of incubation with gentamicn whereas MSDH, the lysosomal detergent used as positive control allow us to detect such change as from 1 hour of incubation. These results have not allowed us to conclude for the involvement of cathepsin D in gentamicin-induced apoptosis. Indeed, if lysosomes are destabilized in presence of gentamicin, other lysosomal constituents such as **gentamicin itself** could be released and **acts directly as**

apoptosis inducer. To test the hypothesis that gentamicin could induce apoptosis once in the cytosol, we have used electroporation technique to deliver gentamicin in the cytosol, by-passing then the lysosomal sequestration and accumulation of this antibiotic as obtained after incubation of cells.

3. GENTAMICIN CAUSES APOPTOSIS AT LOW CONCENTRATIONS IN RENAL LLC-PK_1 CELLS SUBJECTED TO ELECTROPORATION

Until now, we have demonstrated that in gentamicin-induced apoptosis, the first event detected is a lysosomal destabilization (after 2 hours) followed by the activation of mitochondria (after 10 hours) and finally the activation of caspases in parallel to the observation of the DNA condensation and fragmentation (after 24 hours). Based on the literature demonstrating the involvement of lysosomes and the cathepsins, we have previously postulated that cathepsins B and D could be involved in gentamicin-induced apoptosis. By the use of leupeptin, an inhibitor of cathepsin B, we have demonstrated that this cathepsin seemed not involved in gentamicin-induced apoptosis. At the opposite, the addition of pepstatin A, an inhibitor of cathepsin D is protective from gentamicin-induced apoptosis. However, our attempts to test the release of cathepsin D which is crucial for its apoptotic ability have not allowed us to involve or exclude it in gentamicin-induced apoptosis. Further analysis should be made to answer correctly to this question. However, if cathepsins can be released after lysosomal permeabilzation, other lysosomal constituents could also be released. In this context, gentamin appears to us to represent an attracting candidate due to its huge lysosomal accumulation as linker of lysosomal and mitochondrial events (see figure 25 for schematic representation). Indeed, it has been shown that the lysosomal destabilization and release of components is not specific for some lysosomal constituents but rather dependent of the size of the components. Molecules that can be released have been shown to present a molecular size inferior 70 kDa (Bidere *et al.*, 2003).

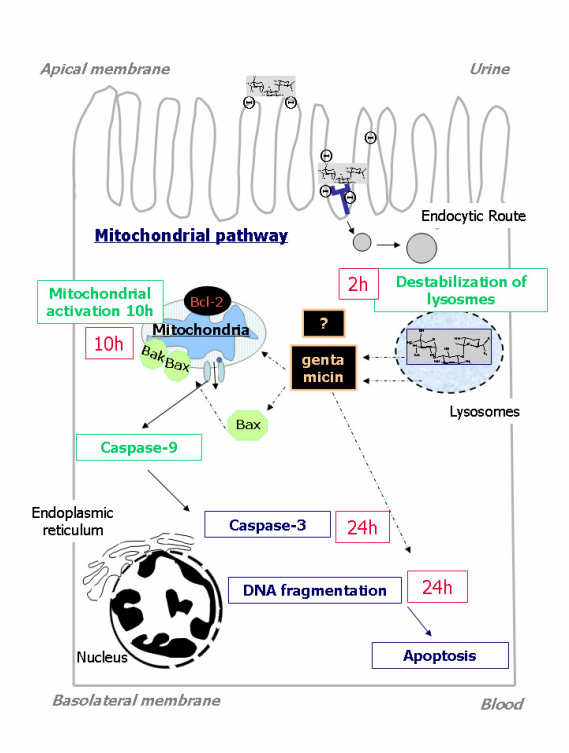

Figure 25: *Schematic representation of the apoptotic alterations observed in a proximal tubular cell exposed to gentamicin (1 to 3 mM) with the hypothesis that gentamicin could constitute the link lysosomal and mitochondrial events.*

To test the possibility that gentamicin can activate apoptosis “outside” the lysosomes, we take advantages of the electroporation technique, which allows introducing some impermeant drugs inside the cell. Doing so, lysosomal event is by-passed and gentamicin is then mainly localised inside the cytosol where its pro-apoptotic ability can be tested.

This hypothesis has been the object of a second paper which is currently in press in Antimicrobial Agents and Chemotherapy.

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Gentamicin Causes Apoptosis at Low Concentrations in Renal LLC-PK$_1$ Cells Subjected to Electroporation

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Gentamicin accumulates in the lysosomes of kidney proximal tubular cells and causes apoptosis at clinically relevant doses. Gentamicin-induced apoptosis can be reproduced with cultured renal cells, but only at high extracellular concentrations (1 to 3 mM; 0.4 to 1.2 g/liter) because of its low level of uptake. We recently showed that gentamicin-induced apoptosis in LLC-PK$_1$ cells involves a rapid (2-h) permeabilization of lysosomes and activation of the mitochondrial pathway of apoptosis (10 h). We now examine whether the delivery of gentamicin to the cytosol by electroporation would sensitize LLC-PK$_1$ cells to apoptosis. Cells were subjected to eight pulses (1 ms) at 800 V/cm (square waves) in the presence of gentamicin (3 μM to 3 mM; 1.2 mg/liter to 1.2 g/liter); returned to gentamicin-free medium; and examined at 8 h for their Bax (a marker of mitochondrial pathway activation) contents by Western blotting and competitive reverse transcriptase PCR and at 24 h for apoptosis by 4′,6′-diamidino-2′-phenylindole staining (confirmed by electron microscopy) and for necrosis (by determination of lactate dehydrogenase release). Nonelectroporated cells were incubated with gentamicin for 8 and 24 h. Significant increases in Bax levels (8 h) and apoptosis (24 h) were detected with 0.03 mM (13.2 mg/liter) gentamicin in electroporated cells compared with those achieved with 2 mM (928 mg/liter) in incubated cells. The increase in the Bax level was not associated with an increase in the level of its mRNA but was associated with the accumulation of ubiquitinated forms (probably as a result of impairment of its degradation by the proteasome). Assay of cell-associated gentamicin showed a marked, immediate, but transient accumulation in electroporated cells, whereas a slow, steady uptake was detected in incubated cells. The data indicate that cytosolic gentamicin triggers apoptosis. Sequestration of gentamicin in lysosomes would, to some extent, protect against apoptosis.

Aminoglycoside antibiotics have long been known to cause acute renal failure in patients, in association with histological and functional signs of proximal tubule toxicity (1). Yet, the underlying molecular mechanisms still remain poorly defined (15). Aminoglycosides enter proximal tubular cells by pinocytosis from the luminal pole and accumulate in lysosomes (18, 27, 62), where they cause a conspicuous phospholipidosis (29, 32). Maneuvers that prevent gentamicin pinocytic uptake (59) and/or the development of lysosomal phospholipidosis (28) protect against nephrotoxicity. Together with the fact that the corresponding morphological and biochemical alterations develop early on in animals or humans treated with clinically relevant doses (10, 32), this was the main argument that linked aminoglycoside cell toxicity to its lysosomal storage and the ensuing organelle dysfunction (40). Gentamicin, indeed, is a highly polar compound and is therefore largely considered to be unable to pass across membranes and to act outside of lysosomes. Besides lysosomal changes, however, the proximal tubules of animals treated with gentamicin also show clear signs of apoptosis (11, 34). This effect of gentamicin, together with the lysosomal alterations described above, can be reproduced in vitro with both renal and nonrenal cells (2, 12). These, however, must be exposed to high concentrations of gentamicin because of poorly efficient drug uptake (12). Using LLC-PK$_1$ cells, an accepted model of renal proximal tubular cells for the study of aminoglycoside toxicity (24, 64, 66, 67, 72), we observed that gentamicin causes an early permeabilization of lysosomes before activation of the mitochondrial pathway of apoptosis can be detected (61). In parallel, recent data indicate that a minor but probably significant proportion of the gentamicin accumulated by cells can reach the cytosol and the mitochondria by trafficking through the Golgi apparatus and the endoplasmic reticulum (56). We also know that apoptosis can be triggered in the kidney by cytosolic signals, such as an increase in the cytosolic Bax protein content, which activates the so-called mitochondrial pathway of apoptosis (54). This has raised the question whether it is not the cytosolic gentamicin that is released from or transported out of lysosomes that triggers apoptosis. This has now been tested directly by taking advantage of the technique of electroporation, which allows the direct delivery of membrane-impermeant solutes into cells (14). Our results show that LLC-PK$_1$ cells undergo extensive apoptosis when they are subjected to electroporation in the presence of concentrations of gentamicin about 100-fold lower than what is necessary if cells are incubated with the drug. We also show that this process is associated with an increase in the cell content of the proapoptogenic protein Bax, which acts upstream of the mitochondria in the cascade that leads to apoptosis (71). The data therefore imply that the lysosomal sequestration of gentamicin could actually protect cells from its apoptogenic effect and thereby mitigate its toxicity.

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MATERIALS AND METHODS

Cells. All experiments were performed with LLC-PK_1 (Lilly Laboratories Culture-pig kidney) cells, clone ATCC CL-101, that originated from and that displayed the attributes of proximal tubular cells (60). They were cultivated in Dubelcco's modified Eagle's medium supplemented with 10% fetal calf serum in an atmosphere of 95% air–5% CO_2; subcultured twice a week; and used at about 80% confluence to avoid cell detachment, which spontaneously takes place after the cells reach confluence. Cells grown under these conditions are not polarized and, in contrast to LLC-PK_1 cells grown on inserts to trigger their polarization (47), do not express megalin (17), a protein that causes receptor-mediated endocytosis of gentamicin in renal tubular cells in vivo (42). Previous studies showed that gentamicin is taken up by fluid endocytosis in LLC-PK_1 cells (i.e., without evidence of membrane binding) under our conditions of culture (12).

Electroporation. We follow the general technique developed for delivery of membrane-impermeant drugs in mammalian cells (14, 26, 53). In brief, subconfluent cells were detached by trypsinization (5 g/liter trypsin, 2 g/liter sodium EDTA, 8. g/liter NaCl), centrifuged at 1,000 rpm (5810 R centrifuge equipped with an A-4-62 rotor; Eppendorf AG, Hamburg, Germany), and resuspended in electroporation buffer (10 mM Na_2HPO_4/KH_2PO_4, pH 7.2, 250 mM sucrose, 1 mM $MgCl_2$) in the presence of a suitable concentration of gentamicin (0 to 3 mM). One hundred microliters of cell suspension (roughly corresponding to 50 μg of cell protein) was placed in electroporation cuvettes (model 620; BTX-Genetronics Inc., San Diego, CA) made of two embedded aluminum electrodes 2 mm apart. The cells were exposed to 8 square-wave pulses (PA-4000 device; Cyto Pulse Sciences, Inc., Columbia, MD) with an electric field of 800 V/cm for 1 ms (with a 1-Hz repetition frequency) and were thereafter left for 15 min at room temperature in the same medium. They were then dispersed in 5 ml of gentamicin-free, complete culture medium and transferred into cell culture dishes for incubation at 37°C for up to 24 h. These conditions were selected based on preliminary pilot studies that assessed the efficiency of the cell penetration of two nonpermeant tracers (trypan blue and lucifer yellow) compared with the release of the cytosolic enzyme lactate dehydrogenase (as a marker of cell membrane leakage). For the sake of conciseness, cells treated according to this protocol are consistently referred to as "electroporated."

Incubation. The cells were continuously exposed to gentamicin at 37°C in complete cell culture medium at the indicated concentrations and for the indicated times. Cells treated according to this protocol are consistently referred to as "incubated."

Detection of apoptosis and necrosis. Apoptotic cells were enumerated after they were stained with 4′,6′-diamidine-2′-phenylindole (DAPI) to reveal the characteristic nuclear changes of apoptosis (condensation and fragmentation of the nuclear material), as described earlier (61). Necrosis was assessed by measuring the amount of the lactate dehydrogenase activity released in the medium (43).

Measurement of gentamicin cell content. Cells were collected by scrapping them into ice-cold phosphate-buffered saline (PBS) after three successive washes with the same buffer and were subjected to brief sonication. Gentamicin was then assayed by a disk-plate microbiological technique using *Bacillus subtilis* (ATCC 6633) as the test organism, as described previously (12, 68). Our routine assay had a limit of detection of 0.1 mg/liter, with a range of linearity up to 64 mg/liter ($R^2 = 0.994 \pm 0.005$ [$n = 6$]). The protein content of the samples was measured by the Folin-Ciocalteu method (38). All gentamicin cell contents were expressed as μg/mg cell protein. The apparent cellular gentamicin concentration was then calculated based on a cell volume of 5 μl per mg protein (68). In previous studies, we showed that samples from control cells spiked with amounts of gentamicin similar to those detected here in electroporated or incubated cells gave readings within 90% of the expected values.

Detection of Bax and ubiquitinated Bax. The cells were washed three times with ice-cold PBS and resuspended in lysis buffer (10 mM *N*-2-hydroxyethyl piperazine-*N*′-2-ethane sulfonic acid [HEPES], 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propane sulfonate [CHAPS], 5 mM 1,4-dithiothreitol [DTT]) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 20 μg/ml leupeptin). The samples were then subjected to five successive cycles of freezing-thawing (dry ice, acetone, 37°C water bath) and centrifuged at 12,000 rpm for 20 min (Eppendorf 5415C centrifuge). The supernatants were collected, and 10 μg of protein (as measured by a modified Bradford method [51]) was mixed with Laemmli buffer for detection of Bax by Western blot analysis, as described previously (61), and by use of a rabbit anti-Bax antibody raised against a synthetic peptide corresponding to amino acids 44 to 62 of human Bax (1:500; Calbiochem Immunochemicals catalog no. 196821; Merck KGaA, Darmstadt, Germany) and peroxidase-labeled anti-rabbit immunoglobulin G (IgG). Bands were revealed by using the SuperSignal West Pico chemiluminescence substrate (Pierce, Rockford, IL). The films were scanned with a commercial flatbed digitizer operated at a resolution of 600 dpi, and the images were subjected to densitometric analysis for the bands corresponding to the monomer of Bax (21 kDa [48]) by using Image J software (version 1.3.1; available from the Research Service Branch of the National Institute of Mental Health [http://rsb.info.nih.gov/ij]). For detection of ubiquitinated Bax, the lysis buffer was supplemented with 1 mg/liter ubiquitin aldehyde and 10 μM Z-Leu-Leu-Leu-aldehyde (MG132; an inhibitor of the proteasome) and protease inhibitors (pepstatin, aprotinin, leupeptin). Fifty micrograms of protein was then immunoprecipitated by addition of 3 μl of undiluted anti-Bax antibody, followed by an overnight incubation at 4°C in a total volume of 1 ml. The mixture was then mixed with 30 μl protein A-agarose beads for 2 h at 4°C and centrifuged at 12,500 rpm (Eppendorf 5415C centrifuge) for 5 min at 4°C. The pellets were washed twice with ice-cold lysis buffer, once in 0.5 M NaCl, and once with PBS and were finally resuspended in Laemmli buffer for Western blot analysis. Ubiquitinated proteins were identified by incubating the membranes for 24 h at 4°C with a mouse monoclonal antibody (FK2; 1:1,000) directed against ubiquitinated proteins (but not free ubiquitin) and obtained from Biomol International LP, Plymouth, PA. Visualization was obtained with peroxidase-labeled anti-mouse IgG, and the bands were revealed and quantified as described above by using the 47- to 55-kDa region corresponding to the ubiquitinated forms of Bax (35, 50).

Quantitative measurement of Bax mRNA. We used the competitive reverse transcriptase PCR (RT-PCR) technique using an internal standard (7). RNA was extracted by using the TRIzol reagent (Life Technologies, Gaithersburg, MD) and treated with 0.8 kU of DNase I (QIAGEN GmbH, Hilden, Germany) for 15 min at room temperature. The RNA was purified with the clean-up RNeasy Mini Kit (QIAGEN), the purity was assessed by spectrophotometry (ratio of absorbance at 260 nm/absorbance at 280 nm in water of at least 1.7 to 1.8), and the quantity of RNA was calculated by determination of its absorbance at 260 nm (assuming a specific absorption coefficient of 0.025 ml μg^{-1} cm^{-1}). cDNA synthesis was carried out with the reverse transcription system of Promega (Promega Corp., Madison, WI) with 5 μg of RNA. The internal standard was prepared by RT-PCR and was designed to be smaller than the target amplicon in the competitive RT-PCR. The forward primer contained an internal sequence of *bax* preceded by a more upstream sequence in the gene (underlined), which corresponds to the full-length *bax* forward primer sequence (underlined) (5′-CAGCT CTGAGCAGATCATGAAGACAGGACAGTAACATGGAGCTGCA-3′), and the sequence shown below as the reverse primer. The PCR product was subjected to agarose gel electrophoresis, and the amplicon (sBax; which was used as the internal standard) was cut and extracted by using a QIAquick gel extraction kit (QIAGEN). The DNA was quantified by performing electrophoresis with the MassRuler DNA ladder (Fermentas International Inc., Burlington, Ontario, Canada). Low-range intensities were assessed by a Bio-Rad Gel Doc analyzer operated with Quantity One software. The estimation of the Bax mRNA concentration was performed by mixing the reverse transcription products of the samples with various amounts of internal standard (from 1 to 1,000 ag) and with the primers (full-length *bax* sequence) 5′-CAGCTCTGAGCAGATCATGAAG ACA-3′ (forward primer) and 5′-GCCCATCTTCTTCCAGATGGTGAGC-3′ (reverse primer) (final primer concentrations, 0.2 μM) with 0.18 mM deoxynucleoside triphosphate mixture in PCR buffer (7.5 mM Tris-HCl [pH 9]), 0.2 mM $MgCl_2$, 5 mM KCl, 2 mM $(NH_4)_2SO_4$, and 2.5 U of polymerase (BioTools B & M Labs, S.A. Madrid, Spain). The PCR profile was 95°C for 45 s, 60°C for 45 s, and 72°C for 2 min for 30 cycles, followed by termination incubation at 72°C for 7 min. After PCR, aliquots of the reaction products were analyzed by electrophoresis in a 1.8% agarose gel. The gel was then stained in electrophoresis buffer containing 0.5 μg/ml ethidium bromide and washed in 1 mM $MgSO_4$. The amount of mRNA was estimated by comparing the relative intensities of the amplified internal standard and of the cDNA of interest (Bax). The relative amounts of DNA corresponding to sBax (internal standard) and Bax were measured by using the Bio-Rad Gel Doc analyzer.

Statistical analyses. The significance of the differences between paired values was tested by Student's *t* test (Excel 2000; Microsoft, Inc., Bellevue, WA). Correlations were calculated by using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA).

Materials. Dulbecco's modification of Eagle's minimum essential medium and trypsin-EDTA were purchased from Life Technologies, Paisley, United Kingdom. Gentamicin sulfate was provided as Geomycine (the branded product registered for clinical use in Belgium) by Glaxo-SmithKline Belgium (on behalf of Schering-Plough Corp., Kenilworth, NJ); conversion of grams to moles was done by using a mean molecular weight of 464.4 (sulfate salt form), based on the average contribution of the main subcomponents (C-1, 30%; C-1a, 20%; C-2, 50%) required by the European Pharmacopoeia (65). Aprotinin, HEPES, CHAPS, PMSF, DTT, ubiquitin-aldehyde, and Z-Leu-Leu-Leu-aldehyde (MG132)

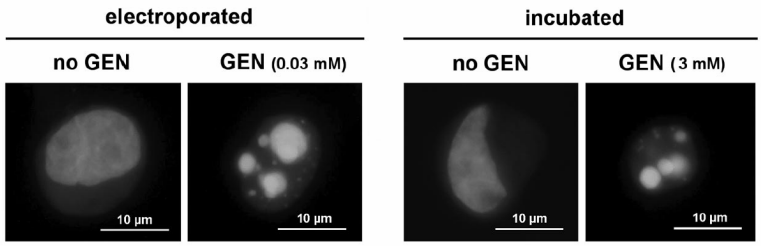

FIG. 1. Staining of nuclei of LLC-PK$_1$ cells by DAPI. Electroporated (left panel), cells were electroporated in the absence of gentamicin (no GEN) or in the presence of gentamicin (GEN) at the concentration shown (0.03 mM; 13.9 mg/liter) and were examined 24 h later; incubated (right panel), cells were maintained for 24 h in the absence of gentamicin (no GEN) or in the presence of gentamicin (GEN) at the concentration shown (3 mM; 1.39 g/liter). In the absence of gentamicin, both electroporated and incubated cells show diffuse finely reticulated staining characteristic of the euchromatin of diploid interphase animal cells. In contrast, cells electroporated or incubated in the presence of gentamicin show typical changes associated with apoptosis, consisting of the condensation and fragmentation of the nuclear material. A color version of this figure is available for download at http://www.facm.ucl.ac.be/public/AAC-0897-05-Figure-1-color.jpg.

were purchased from Sigma-Aldrich (St. Louis, MO). DAPI and protein A-agarose beads came from Roche Diagnostics (F. Hoffmann-La Roche Ltd., Basel, Switzerland).

RESULTS

Validation of electroporation conditions. The efficacy of electroporation for the delivery of nonpermeant solutes was assessed by examining the penetration of trypan blue and lucifer yellow added to the electroporation buffer. Fifteen minutes after electroporation, 64.3% ± 6.5% (trypan blue) and 63.4% ± 6.0% (lucifer yellow) of the cells were homogeneously stained. In parallel, the effective resealing of the pericellular membrane was assessed through the measurement of the release of the cytosolic enzyme lactate dehydrogenase into the culture medium. A typical value of release, recorded 15 min after electroporation, was 11.3% ± 2.1% of the total cell content, whereas the value was 9.1% ± 2.3% for nonelectroporated cells. Electroporated cells were also examined under an electron microscope to verify the absence of alterations of the main subcellular organelles and the intactness of the pericellular membrane. No significant difference from the control (nonelectroporated) cells was noticed.

Morphological demonstration of apoptosis. Cells subjected to electroporation in the presence of increasing concentrations of gentamicin were examined 24 h after electroporation for morphological evidence of apoptosis (DAPI staining). As shown in Fig. 1, cells electroporated in the presence of low concentrations of gentamicin (0.03 mM; 13.9 mg/liter) already showed typical images of apoptosis, consisting of the fragmentation and condensation of the nuclear material. These images were similar to those seen for cells incubated with gentamicin for 24 h but at considerably higher concentrations (3 mM; 1.39 g/liter), as described previously (61). The occurrence of apoptosis in cells electroporated in the presence of low concentrations of gentamicin was confirmed by electron microscopy, which revealed typical images, such as the condensation of chromatin material abutting the nuclear membrane (Fig. 2). These cells did not show a visible alteration of their pericellular membrane (inset of Fig. 2). Similar images were seen in the present investigation for cells incubated with gentamicin at high concentrations (1 to 3 mM; data not shown) and in our previous study (12).

Quantitative analysis of apoptosis. Figure 3 shows the percentage of apoptotic cells (as assessed by DAPI staining) among cells electroporated in the presence of increasing concentrations of gentamicin and examined after 24 h of culture in drug-free medium in comparison with that among cells incubated for 24 h with increasing concentrations of gentamicin. It appears that the electroporated cells were about 100-fold more sensitive to gentamicin-induced apoptosis than the incubated cells. The frequency of apoptotic cells among the electroporated cells, however, reached a maximum at 0.3 mM (139 mg/liter) and sharply declined thereafter. With high gentamicin concentrations, indeed, most samples showed a large proportion of cells undergoing pycnotic nuclear degeneration (characterized by highly condensed but not fragmented nuclear material), suggesting the occurrence of massive necrosis. This was confirmed by measurement of the release of lactate dehydrogenase, as shown in the lower panel of Fig. 3. Cells incubated with gentamicin did not show necrosis within the range of concentrations investigated here (up to 3 mM [1.39 g/liter]). Yet, incubation of the cells with higher concentrations (from 4 mM [1.85 g/liter]) caused detectable necrosis (data not shown), as reported previously (61).

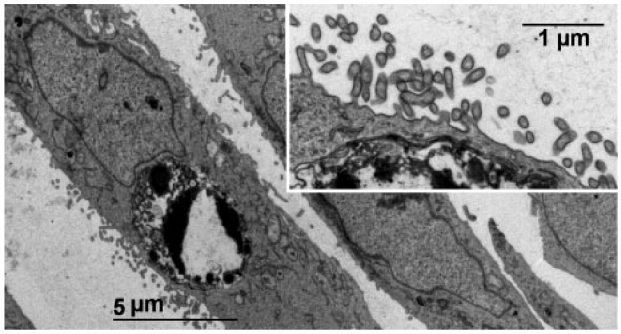

FIG. 2. Electron microscopy of LLC-PK$_1$ cells electroporated in the presence of 0.1 mM (46.4 mg/liter) gentamicin and examined 24 h later. The main image shows a typical nucleus demonstrating apoptotic features consisting of condensed and fragmented chromatin clumps abutting the perinuclear membrane. The inset shows that the pericellular membrane of that cell is intact (with many, normal-looking microvilli).

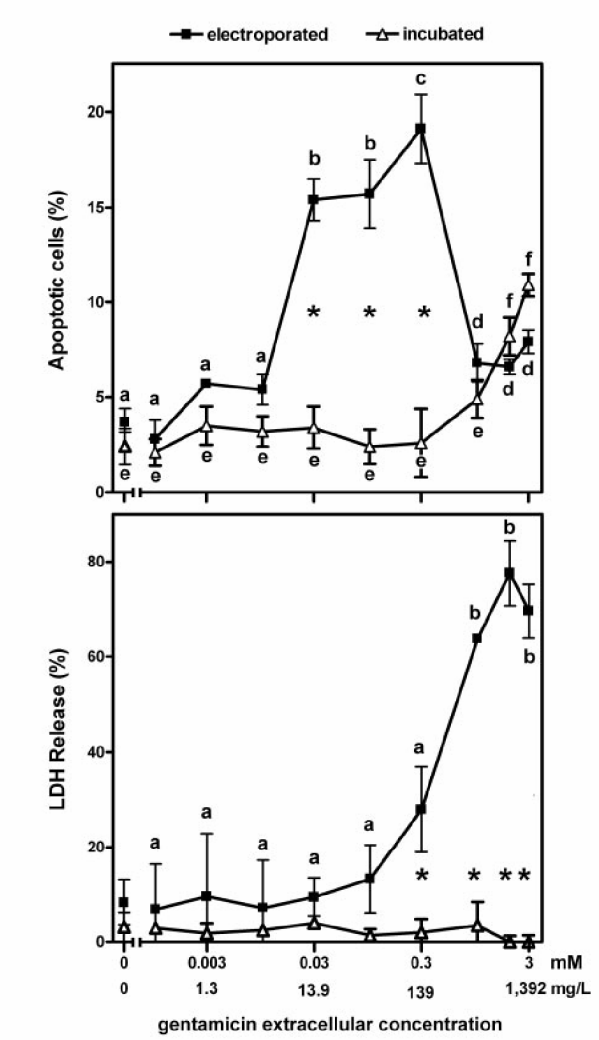

FIG. 3. Influence of gentamicin concentration on apoptosis (upper panel) and necrosis (lower panel) in LLC-PK_1 cells. Apoptosis was assessed by enumeration of typical apoptotic nuclei (DAPI staining; see Fig. 1). Necrosis was assessed by measuring the release of the cytosolic enzyme lactate dehydrogenase (LDH) in the culture medium (shown as the percentage of the total amount in cells plus medium). Electroporated, cells were subjected to electroporation in the presence of gentamicin at the concentrations showed in the abcissa, returned to gentamicin-free medium, and examined 24 h later; incubated, cells were cultivated for 24 h in the presence of gentamicin at the concentrations shown on the abscissa. The 0 value on the abscissa corresponds to cells electroporated (closed symbols) or incubated (open symbols) in the absence of gentamicin. Values are means ± standard deviations ($n = 3$). Statistical analysis was performed by the use of analysis of variance, and the values of the datum points with different letters in the electroporated cell or the incubated cell panels are significantly different from those of all other points in the same group ($P < 0.05$; for lactate dehydrogenase release [lower panel], there is no significant difference among any of the datum points for incubated cells); the asterisks show the significant differences ($P < 0.05$) between groups (electroporated versus incubated cells).

Penetration and retention of gentamicin in cells. Figure 4 shows the variation of the apparent cellular gentamicin content in cells electroporated in the presence of the drug (at increasing concentrations) and monitored for the next 24 h compared with that in cells incubated with increasing concentrations of the drug for up to 24 h (while the two processes are very different, we present the data together since comparison of the data is instructive). With electroporated cells (left panel of Fig. 4), there was a marked and immediate accumulation of gentamicin that was proportional to its extracellular concentration, with an apparent cellular concentration to extracellular concentration ratio of approximately 6 (see the slope of the correlation shown in the inset of the left panel of Fig. 4). The amount of gentamicin found to be associated with electroporated cells exposed to high extracellular concentrations of gentamicin (0.3 mM [139 mg/liter] or higher) quickly decreased over the next 6 h and decreased more slowly thereafter to reach values about 8- to 10-fold lower than the original ones after 24 h. In contrast, the amount of gentamicin found in cells electroporated in the presence of 0.1 mM (46.4 mg/liter) gentamicin decreased to only about 30% of the original amount within 24 h. No decrease was seen from cells incubated with only 0.03 mM gentamicin (13.9 mg/liter). This behavior was in sharp contrast to what was observed with cells incubated with gentamicin (right panel of Fig. 4). In accordance with our previous results (12), the uptake of gentamicin proceeded almost linearly with time and was proportional to the extracellular concentration (resulting in a mean, stable clearance of 4.44 μl per mg of cell protein and per day). The apparent cellular concentration to extracellular concentration ratio of gentamicin in incubated cells was about 0.8 after 24 h (see the slope of the correlation shown in the inset of the right panel of Fig. 4), in accordance with the findings of our previous studies (12).

It is interesting that cells electroporated in the presence of 0.03 mM (13.9 mg/liter) gentamicin showed an apparent drug concentration (measured after 1 h) of about 0.3 μg/mg protein and that these cells developed extensive apoptosis (16%) within the next 24 h. By comparison, in this time frame, cells incubated for 24 h with 3 mM gentamicin developed about 11% apoptosis, for an apparent cell gentamicin content of about 5.4 μg/mg when it was measured at 24 h.

Increase in the cell content of the proapoptotic Bax protein. Apoptosis induced by cytotoxic agents is increasingly reported to be associated with a larger cell content of the proapoptotic protein Bax (4, 52, 57). In pilot studies with cells incubated with gentamicin (2 mM), we observed a significant increase in the level of Bax from 8 h of incubation, and this progressed over time up to 32 h. We therefore looked for a similar increase in cells electroporated in the presence of gentamicin. Figure 5 shows that there was a marked increase in the cell content of Bax 8 h after electroporation at extracellular gentamicin concentrations of 0.03 and 0.1 mM. At 0.03 mM, this increase (about twofold) was similar to that seen with cells incubated for 8 h with 2 mM gentamicin. The origin of this increase was then examined in terms of Bax overexpression and/or decreased degradation. Competitive RT-PCR failed to reveal significant changes in the cell content of Bax mRNA (data not shown). In contrast, Fig. 6 shows that the ubiquitinated forms of Bax were significantly increased in cells electroporated in the presence of gentamicin as well as in cells incubated with gentamicin, largely in parallel with the increases in Bax itself.

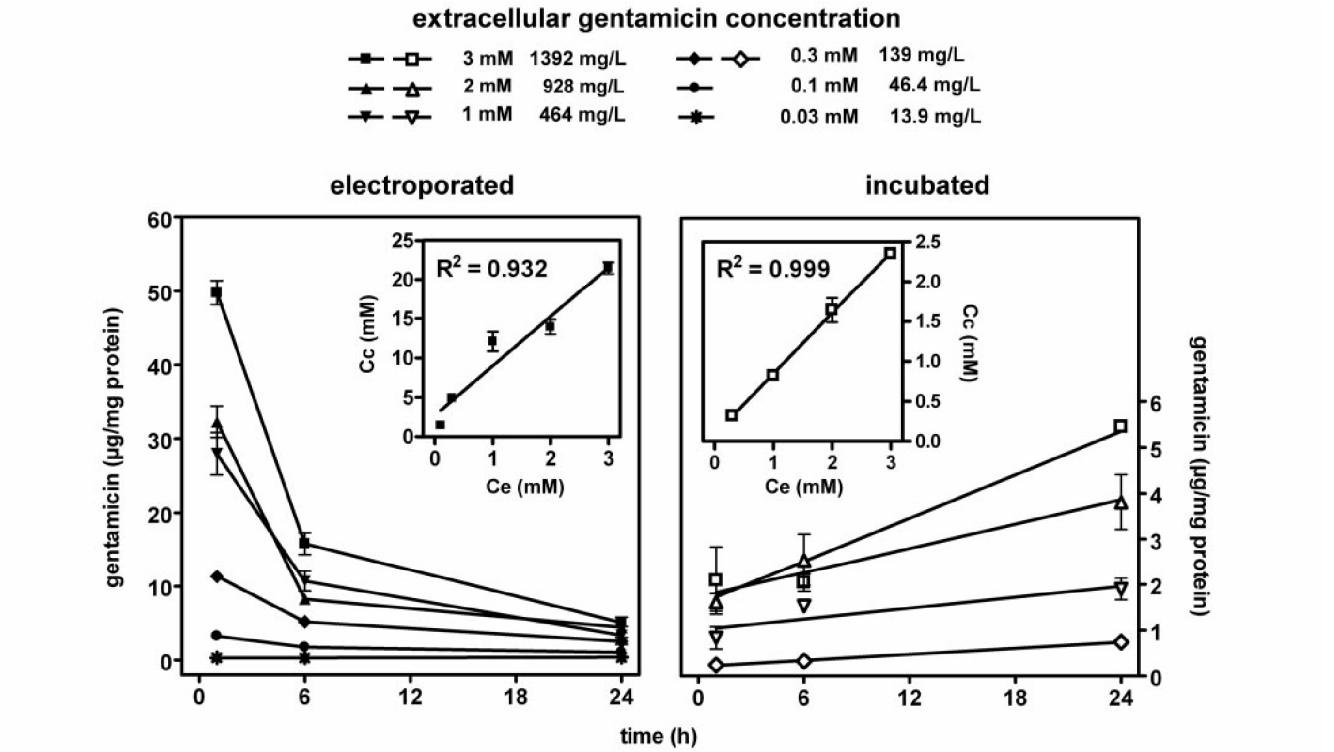

FIG. 4. Cell contents in gentamicin as a function of its extracellular concentration and time. Electroporated (left panel), cells were subjected to electroporation in the presence of gentamicin at the concentrations indicated by the closed symbols at the top of the graphs and returned to drug-free medium for incubation at 37°C for the times indicated on the abscissa; incubated (right panel), cells were incubated at 37°C in the presence of gentamicin at the concentrations indicated by the open symbols at the top of the graphs for the times indicated on the abscissa. Values are means ± standard deviations (n = 3). (Insets) Correlation between the apparent cellular concentration (Cc) and the extracellular concentration (Ce) measured 1 h after electroporation for electroporated cells (left; slope, 6.3 ± 0.5) and after 24 h for incubated cells (right; slope, 0.76 ± 0.02).

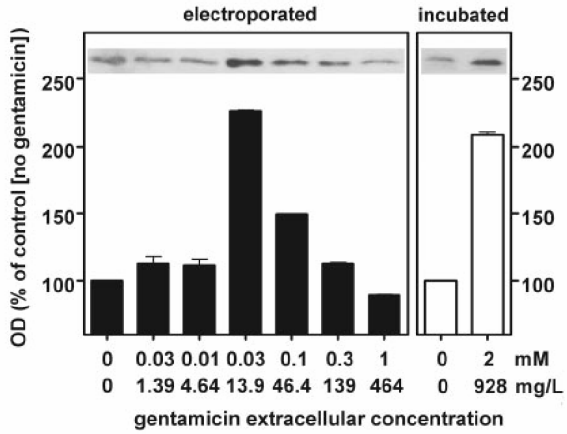

FIG. 5. Detection of the proapoptotic protein Bax by Western blot analysis of lysates of LLC-PK$_1$ cells. The films were analyzed by densitometry (with measurements done in triplicate; the values shown are means ± standard deviations; when not visible, the standard deviation bars are smaller than the minimal resolution of the graph). Electroporated (left panel), cells were subjected to electroporation in the presence of gentamicin at the concentrations shown on the abscissa and returned to drug-free medium for 8 h at 37°C before they were collected and processed; incubated (right panel), cells were maintained at 37°C for 8 h in the presence of gentamicin at the concentrations indicated on the abscissa. The 0 value on the abscissa corresponds to cells electroporated (closed bars) or incubated (open bars) in the absence of gentamicin. OD, optical density.

DISCUSSION

The main finding of the present study is that gentamicin causes apoptosis in LLC-PK$_1$ cells when they are subjected to electroporation with extracellular drug concentrations considerably lower than those needed to cause apoptosis in cells incubated with gentamicin. These concentrations (10 to 15 mg/liter) are both microbiologically and pharmacologically meaningful (12, 61). This takes place without evidence of gross cell damage (beyond what could be associated with apoptosis), based on morphological data and a lack of marked lactate dehydrogenase release. The absence of visible alterations of intracellular organelles upon electroporation is consistent with the fact that the effect of electric pulses on membrane permeability is critically dependent on the size of the corresponding structures. Lysosomes and mitochondria, for instance, typically require much more severe conditions than whole cells, namely, a 300-fold higher potential and much shorter pulse durations, to be significantly affected by electroporation (3).

Although considered a valid in vitro model for the study of aminoglycoside-induced toxicity, LLC-PK$_1$ cells accumulate gentamicin much more slowly and less effectively than proximal tubular cells. As observed here and as discussed earlier (12), the rate of gentamicin uptake in LLC-PK$_1$ cells cultivated as monolayers is indeed similar to that of validated tracers of fluid-phase pinocytosis. The situation is different in proximal

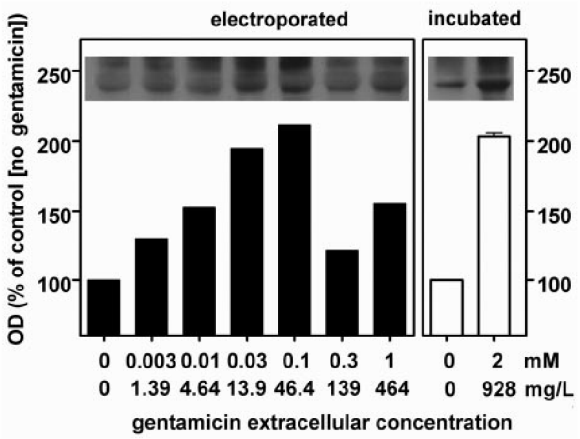

FIG. 6. Detection of the ubiquitinated forms of the proapoptotic protein Bax by Western blot analysis of lysates of LLC-PK_1 cells. Bax was immunoprecipitated with an anti-Bax antibody, and the uniquitinated proteins were revealed on the membrane with an antibody directed against ubiquitinated proteins but not free ubiquitin. The films were analyzed by densitometry measurements made in triplicate; the values shown are means ± standard deviations; when not visible, the standard deviation bars are smaller than the minimal resolution of the graph). Electroporated (left panel), cells were subjected to electroporation in the presence of gentamicin at the concentrations shown on the abscissa and then returned to drug-free medium for 8 h at 37°C before they were collected and processed; incubated (right panel), cells were maintained at 37°C for 8 h in the presence of gentamicin at the concentrations indicated on the abscissa. The 0 value on the abscissa corresponds to cells electroporated (closed bars) or incubated (open bars) in the absence of gentamicin. OD, optical density.

tubular cells, where uptake proceeds through adsorptive pinocytosis because of the presence of acidic phospholipids (58) and of a specific binding site for polybasic compounds (megalin [45]) at the level of the brush border of proximal tubular cells. LLC-PK_1 cells, however, seem to express megalin only when they are grown on inserts, i.e., under conditions that allow their polarization (47). Yet, apoptosis can be unambiguously demonstrated in LLC-PK_1 cells grown as monolayers when the cellular concentration of gentamicin is made similar to that observed in the proximal tubules of animals treated with low doses of gentamicin (11), i.e., in a 2- to 20-μg/mg protein range. In the first analysis, it would seem to be reasonable to assume that electroporation simply allows the initial cell concentration of gentamicin to immediately reach the critical cell concentrations needed to trigger apoptosis.

There is, however, a major qualitative difference between gentamicin delivery to cells by pinocytosis (fluid or adsorptive) and delivery by electroporation. Pinocytosis (in cultured cells in vitro or in proximal tubular cells in vivo) should, indeed, direct the drug primarily to lysosomes, as observed for impermeant solutes (5) and for gentamicin in particular (18, 68). In contrast, the data obtained with trypan blue and lucifer yellow indicate that electroporation could allow gentamicin to directly gain access to the cytosol. Yet, we showed that incubation of LLC-PK_1 cells with gentamicin causes a destabilization of lysosomes before the activation of the so-called apoptosis mitochondrial pathway becomes evident (61). This led us to conclude that it is the gentamicin leaking from lysosomes that eventually causes apoptosis in cells incubated with this drug (61).

The role of lysosome destabilization in the initiation of the apoptotic process was further surmised from the fact that gentamicin (i) is able to permeabilize phospholipid bilayers under conditions that mimic the lysosomal environment (61) and (ii) has been shown, from about 10% of the total cell content, to quickly traffic through to the Golgi complex and the endoplasmic reticulum, from where it could be transported to the cytosol and reach mitochondria (56). The higher susceptibility of electroporated cells than incubated cells to apoptosis could therefore result from differences in the subcellular distribution of the bulk of the drug. Quantitative analysis based on the data presented in Fig. 3 and 4 supports this conclusion. Cells electroporated in the presence of gentamicin at 0.03 mM (13.9 mg/liter) showed an apparent drug concentration (measured after 1 h) of about 0.3 μg/mg protein, and these cells developed extensive apoptosis (16%) within the next 24 h. In this time frame, cells incubated for 24 h with 3 mM gentamicin develop about 11% apoptosis, for an apparent cell gentamicin content of about 5.4 μg/mg protein when the content is measured at 24 h. Only 5 to 6% of this amount would need to be delivered to the cytosol during the 24-h incubation period to create a condition similar to that which is probably taking place initially in electroporated cells. This value is close to that which has been suggested to be diverted from the lysosomes of cells incubated with gentamicin, based on a detailed morphological study of its disposition in LLC-PK_1 cells (56).

Such a small proportion of the total amount of gentamicin taken up by cells could not be detected by the cell fractionation techniques previously used to assess the intracellular fate of gentamicin in cultured cells (68) or in the kidney cortex (18). Our study gives no explanation for the decrease in the gentamicin cell content from electroporated cells upon incubation, as seen in Fig. 4. A plausible explanation for the rapid loss of gentamicin from electroporated cells, however, could simply be the nonimmediate closure of the pores and, for cells electroporated with high concentrations of gentamicin and/or that have taken up a large amount of the drug, the onset of necrosis (as observed in Fig. 3). This should be studied in the future, but the limited size of the samples (due to technical constraints linked to the electroporation technique) will require the use of very sensitive and reliable techniques to examine in detail the disposition of the drug and its fate after electroporation.

With respect to the drug found to be associated with the cells after electroporation, we also ignored the subcellular distribution and the proportion that is free. Gentamicin is known to bind to a variety of acidic macromolecules and supramolecular structures, which, incidentally, would explain why its concentration may initially be higher in electroporated cells than in the extracellular fluid. However, binding to nontarget constituents should similarly affect the drug leaking from lysosomes and that entering cells by electroporation.

A direct effect of cytosolic gentamicin as a trigger of apoptosis (irrespective of its origin) is actually strongly suggested from the observation that the cell content of the proapoptotic protein Bax is increased in both electroporated and incubated cells when gentamicin is present. Bax has a pivotal role in controlling apoptosis; and an increase of its cellular content triggers apoptosis, as observed in both cancerous and noncancerous cell lines as well as in vivo, arguing for a non-cell-type-dependent effect (13, 23, 36).

During apoptosis, Bax is indeed known to translocate to the mitochondrial membrane (73) and to cause cytochrome *c* release, which amplifies the apoptotic signal (31). We see here that what differentiates electroporated cells from incubated cells is their greater sensitivity to the increase in the amount of Bax with respect to the gentamicin concentration. We also know that mitochondria are involved in gentamicin-induced apoptosis in LLC-PK_1 cells when these cells are incubated with the drug (61). We may therefore reasonably assume that what triggers apoptosis in electroporated cells is also the increase in the Bax content. Unfortunately, the small amount of cells that could be subjected to electroporation has prevented us from performing fractionation studies to demonstrate directly the translocation of Bax and the release of cytochrome *c*.

As is the case for any protein, the increase in Bax content could result from an increase in its transcription or a decrease in its degradation, or both. The first mechanism is unlikely, since we did not observe significant changes in Bax mRNA levels. Conversely, we observed an increase in the amount of its ubiquitinated forms. Bax is degraded by the proteasome (8, 35) but, like other proteins, needs to be ubiquitinated before it can be processed (specific signaling [21]). Inhibition of the proteasome by specific inhibitors is known to cause an increase in ubiquitinated Bax, and this was shown to slow the rate at which Bax itself can be ubiquitinated (9, 30, 35, 50). Our observations are consistent with such a process but do not identify the nature of the proteasome inhibitor. Yet, a recent study showed that gentamicin may interact directly with the β type 9 subunit of the proteasome (22). The implications of this interaction in terms of modulation of proteolytic activity need, however, to be ascertained.

An apparent difficulty for giving to Bax the central role that we propose, however, is that maximum apoptosis is seen with 0.3 mM gentamicin, whereas the Bax content is maximal at a 10-fold lower gentamicin concentration (0.03 mM). This may result from the combination of three phenomena. First, apoptosis is not linearly related to the concentration of gentamicin. Second, the increase in the amount of Bax with respect to the concentration of an apoptosis-triggering agent is also not linear (a typical example has been presented elsewhere [20]). Third, an increase in the level of Bax precedes apoptosis, making the two processes not necessarily linearly related. Finally, we cannot exclude the possibility that gentamicin itself participates in the triggering of apoptosis (through a direct interaction with the mitochondria, once its concentration reaches a sufficient value, as has been observed in vitro [39]), causing an increase in the level of Bax and apoptosis to become dissociated with respect to the gentamicin concentration.

Many studies indicate that it is the ratio of the proapoptotic Bax and the antiapoptotic Bcl-2 that is critical in the initiation of drug-induced apoptotic cell death (75). We observed previously that Bcl-2 overexpression in LLC-PK_1 cells (through transfection) prevents gentamicin-induced apoptosis (12). We did not examine this possibility here because (i) while Bcl-2 is abundant in embryonic kidney and is expressed in correlation with the apoptosis-related remodeling processes typical of this developmental stage, its expression in adult renal cells is low (63); and (ii) our attempts to detect Bcl-2 in the strain of LLC-PK_1 cells used here revealed only minimal expression by Western blotting (12) or RT-PCR analysis (H. Servais, unpublished data). Yet, others have clearly observed such an expression in LLC-PK_1 cells and have demonstrated its role in protecting the cell from apoptosis in at least two situations of drug-induced nephropathy (46, 74). The literature, however, suggests that rather than Bcl-2, it is its homologue, Bcl-xL, that may be controlling apoptosis in renal cells (37, 49). Future work is therefore needed to better ascertain the role of Bcl-2 and its homologues in gentamicin-induced apoptosis.

Our study also revealed that electroporation in the presence of gentamicin may cause necrosis if the drug concentration exceeds 0.3 mM. This toxic effect is not specifically linked to the electroporation since extensive necrosis is also observed for cells incubated with gentamicin if its extracellular concentration exceeds 3 mM (61). It illustrates the well-known fact that apoptosis and necrosis are two adverse effects that drugs cause at low and high concentrations, respectively (6, 19, 70). This explains why the cell Bax content decreases at high gentamicin concentrations through global, necrosis-related impairment of protein synthesis in association with the fast turnover (1 to 2 h) of that protein (35). In the case of aminoglycosides, apoptosis readily develops in the rat kidney cortex at low doses (11), together with other ultrastructural and functional alterations (for a review, see reference 69). Necrosis, however, becomes systematically predominant at high doses (for a review, see reference 40). The point of interest here is that necrosis could easily be obtained when cells were exposed to gentamicin during electroporation. As discussed earlier, this mode of delivery bypasses the lysosomal sequestration of gentamicin seen in incubated cells or in vivo as a result of its pinocytotic uptake. Thus, contrary to our previous hypotheses, it is possible that lysosomal damage (for a review, see reference 33) and necrosis are unrelated events. The former would be directly related to the local effects of gentamicin, such as phospholipase inhibition (32), while the latter would result from a direct effect of gentamicin on nonlysosomal constituents. The evidence presented here as well as in previous work may even suggest that gentamicin sequestration in lysosomes is, to some extent, protective against apoptosis and necrosis-related toxicities. The observation that polyaspartic acid protects against gentamicin-induced nephrotoxicity (16) by trapping the drug in lysosomes as a nondiffusible ionic complex (28) is also consistent with the view that the lysosomal sequestration of gentamicin is a favorable event as far as nonlysosomal effects are concerned. It remains true, however, that pinocytotic uptake of aminoglycosides by proximal tubular cells is the primary cause of nephrotoxicity, since (i) mice with genetic or functional megalin deficiency, which makes them unable to accumulate the drug in these cells by receptor-mediated pinocytosis, are protected against toxicity (59); and (ii) maneuvers that result in the decreased uptake of aminoglycosides by impairing or saturating their pinocytic uptake are associated with a reduced toxicity (for a review, see reference 40). The lysosomal accumulation of gentamicin would therefore appear to be a double-edged pharmacokinetic weapon for the cell. It would indeed afford protection from general cellular toxicity, as long as lysosomes remain intact. However, it would also precipitate cell death, once gentamicin, stored in large amounts in these organelles, is released as a consequence of drug and phospholipid overloading (33), direct permeabilization of the lysosomal membrane (61), interference with proteins that stabilize the lysosomal

membrane (41), or retrograde trafficking trough the Golgi apparatus and the endoplasmic reticulum (55, 56). Protection against gentamicin-induced nephrotoxicity in vivo could therefore be envisaged not only by blocking its pinocytic uptake in cells (44) but also by preventing its release from lysosomes.

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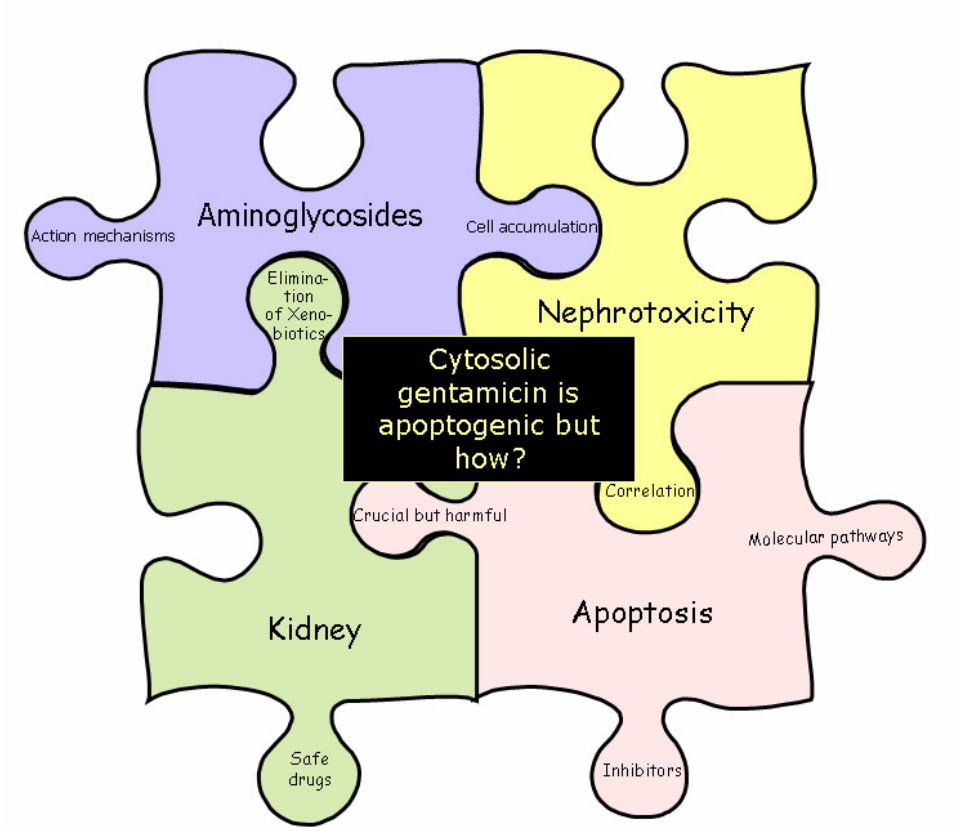
Chapitre IV: CONCLUSIONS AND GENERAL DISCUSSION :



1. MAIN FINDINGS OF THIS WORK

Aminoglycosides antibiotic such as gentamicin are commonly used throughout the world due to their low cost, high effectiveness and low rate of resistance. They are active against a wide range of Gram-negative bacteria as well as against *Staphylococcus aureus*, *Pseudomonas aeruginosa* or *Escherichia coli* and they are primarily used to treat life-threatening infections. However their use is severely hampered by the risk of serious side effect, although a reduction in nephrotoxicity has been achieved by the introduction of optimized dosing regiments. All attempts to chemically modify the drugs to prevent nephrotoxicity have so far been unsuccessful. This is mainly due to the difficulty to dissociate toxicity and antibacterial activity since their positive charge are responsible for their binding to megalin (receptor protein responsible for their accumulation inside kidney cells of the proximal tubule) but are also important for the aminoglycosides entry into the bacteria and for their interaction with the ribosomal complex (and hence bactericidal activity).

The discovery that these antibiotics are able to induce apoptosis at doses corresponding to therapeutic drug levels has brought new elements for the identification of the mechanisms responsible of the aminoglycoside nephrotoxicity (El Mouedden *et al.*, 2000b; El Mouedden *et al.*, 2000a). This should contribute to the development of new approaches whose aim is to reduce the main side effect of the aminoglycosides.

In our study, we have demonstrated that the first apoptosis-related event observed after exposure of LLC-PK1 cells to gentamicin is a destabilization of the lysosomes. This has been observed after 2 hours of contact with the antibiotic. After 8 hours, the cellular amount of cytosolic pro-apoptotic Bax increases, probably due proteasomal dysfunction. Bax has been shown to be an important mediator of the mitochondrial activation, which has been detected as from 10 hours of contact with the antibiotic. This mitochondria activation has

been followed by the release of cytochrome c and the activation of caspase-9, both of which have been shown after 12 hours. Finally, the activation of the effector caspase, caspase-3, was observed in parallel to the nuclear condensation and fragmentation. Figure 26 illustrates all the observations made by our studies.

Based on these observations and on the literature describing the relation between lysosomal destabilization and mitochondrial activation, we first thought that cathepsins would certainly represent an attracting “linker” between lysosomal and mitochondrial events. However, our unexpected but rather interesting observations that only low amounts of gentamicin delivered inside the cellular cytosol of LLC-PK1 cells by electroporation (and thus by-passing the lysosomal sequestration) are able to induce apoptosis has challenged our first hypothesis but also previous idea that lysosomal alterations should be related to cell death of proximal tubular renal cells. Indeed, applying extracellular concentration (0.03 mM or 13.9 mg/L) of about a hundred-time inferior to those needed for incubation is sufficient to induce apoptosis. These observations allow us to conclude that sequestration of aminoglycosides in the lysosomes should more probably be a rather protective event by preventing large quantities of toxic drugs to access the cytosol where they are highly harmful. Some of the lysosomes are nevertheless destabilized resulting either from the overload of drugs and/or lipids (Laurent *et al.*, 1990), or from direct permeabilization by aminoglycosides (Servais *et al.*, 2005), or from the interaction of aminoglycosides with chaperones that contribute to lysosomes functions and stability (Miyazaki *et al.*, 2004; Nylandsted *et al.*, 2004).

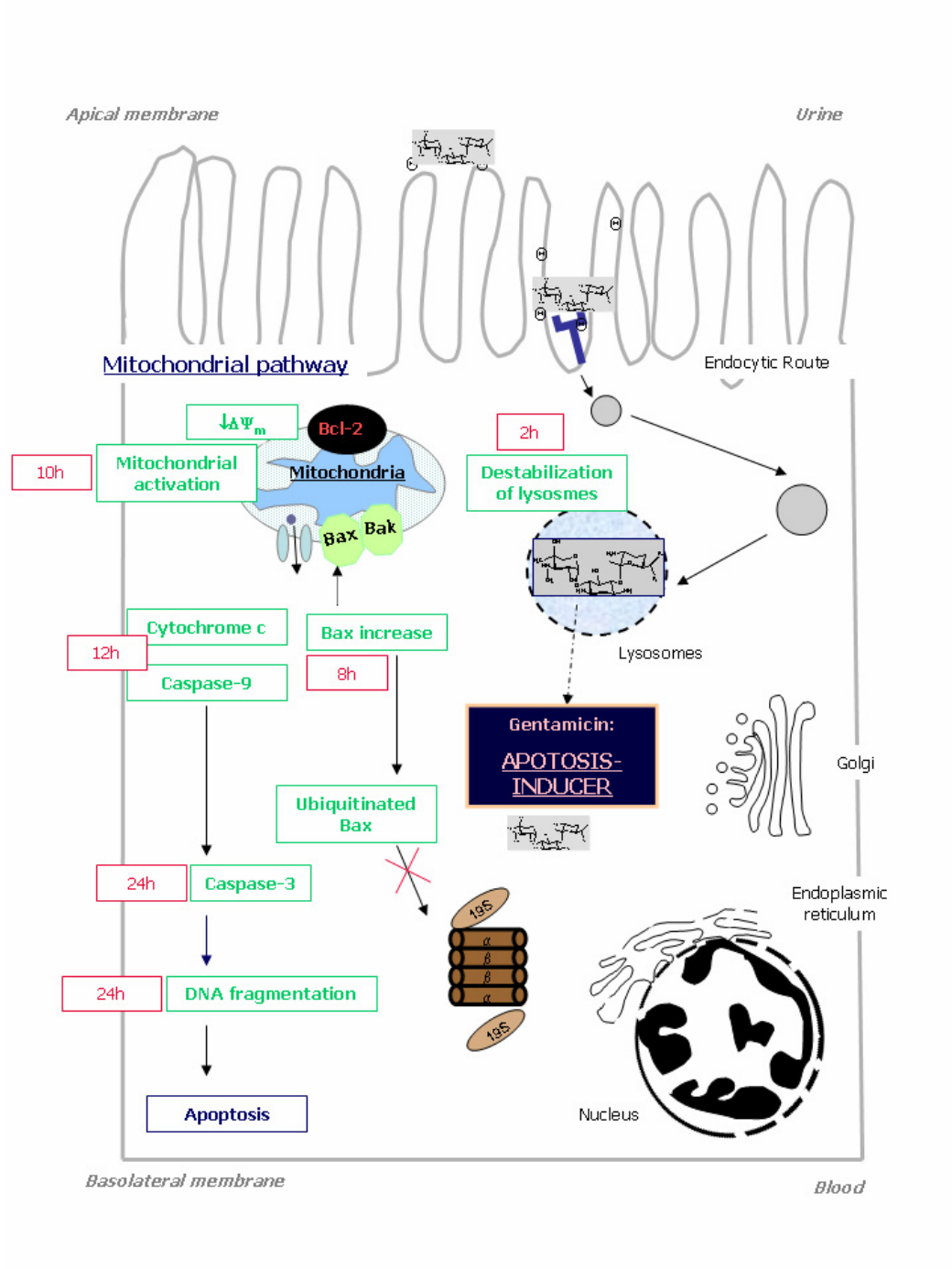


Figure 26: *Resume of the apoptosis-related events induced by gentamicin in LLC-PK1 cells.*

2. MAIN ISSUES ARISING FROM OUR OBSERVATIONS

2.1. Are LLC-PK1 cells an appropriate cellular model?

LLC-PK1 cell line is a porcine kidney cell line (Hull *et al.*, 1976) possessing characteristics of proximal tubule (such as Na^+-dependent transport systems, enzymes located in the apical membrane including alkaline phosphatase and γ-glutamyl transpeptidase) and an electric resistance of the monolayer of 127 $\Omega.cm^2$ that is similar to that of a leaky epithelia (Rabito, 1986). This well characterized cell line has also been widely used to analyze nephrotoxicity of drugs and particularly to study gentamicin nephrotoxicity (Hori *et al.*, 1984; Kohlhepp *et al.*, 1994; Sandoval *et al.*, 1998; Schwertz *et al.*, 1986) but also to study drugs or toxins-induced apoptosis (Nagothu *et al.*, 2005; Gennari *et al.*, 2004; Okuda *et al.*, 2000; Healy *et al.*, 1998). The ability of aminoglycosides to induce apoptosis in renal cell line has also been characterized previously in our lab in LLC-PK1 and MDCK (El Mouedden *et al.*, 2000b). Based on all these works, LLC-PK1 seems to be an appropriate model to study gentamicin-induced apoptosis. However, as it has been shown more recently that gentamicin is taken up by renal proximal tubular cells by megalin-mediated endocytosis and as accumulation in cells is critical for gentamicin toxicity, does LLC-PK1 express megalin? If we check on the literature, some authors have described an expression of megalin in LLC-PK1 cells (Nielsen *et al.*, 1998; Nielsen *et al.*, 2001), where cells have been grown on inserts (and thus polarized) whereas others have not found megalin expression (Decorti *et al.*, 1999; Girton *et al.*, 2002) if using culture conditions similar to ours (grown as monolayers). As we have not specifically checked the expression of megalin in our cell line, we can assume that megalin is not involved in gentamicin uptake in our conditions of cell culture. This was also supported by our observations that gentamicin uptake is non-saturable process and that the clearance of gentamicin from medium (4.44µl/mg protein/24 hours) is similar to that of a typical marker of fluid-phase endocytosis (El Mouedden *et al.*, 2000b; Cupers *et*

al., 1994). The presence of megalin could raise different questions, about the kinetic of gentamicin uptake but also about the availability of gentamicin in lysosomes. For this last question, we could assume that gentamicin can be detached from its receptor in lysosomes since recycling of the receptor occurs rapidly in the endosomes (the acidic environment triggers release of ligand from its receptor) and thus only few megalin could be found in lysosomes for degradation (Gaborik and Hunyady, 2004). This was indeed supported by the observations made by Nielsen and colleagues that have shown the presence of megalin mainly in brush border membranes and in the endosomal compartment of LLC-PK1 cells grown on inserts whereas only few lysosomes were labelled for megalin (Nielsen *et al.*, 1998). Thus, the analysis of the mechanisms by which gentamicin induces apoptosis in cells expressing megalin (LLC-PK1 cells grown on insert or primary culture of human proximal tubular cells) and comparison with our observations could certainly give us additional informations about the events underlying gentamicin nephrotoxicty and certainly more relevance to the *in vivo* situations.

2.2. Could lysosomal destabilization be responsible of gentamicin delivery into the cytosol of incubated cells?

In the first part of our work, we have demonstrated using acridine orange as lysosomal integrity probe that these intracellular organites were rapidly destabilized as from 2 hours of contact with gentamicin and this event was indeed already suspected earlier (see discussion (El Mouedden *et al.*, 2000b; El Mouedden *et al.*, 2000a)). Lysosomal permeabilization has been shown to be associated with the release of some lysosomal constituents such as cathepsins, cathepsins B and D being the major ones involved in apoptosis. The degree of lysosomal permeabilization seemed to be an important parameter leading to cell death (1) by apoptosis for moderate permeabilization and (2) by necrosis for massive permeabilization. Lysosomal permeabilization associated with apoptosis seemed to be a non-selective process, since not only cathepsins D

(Roberg *et al.*, 1999) and cathepsin B (Werneburg *et al.*, 2002) but also lactoferrin (Katunuma *et al.*, 2004), and different lysosomal tracers acridine orange (Servais *et al.*, 2005; Li *et al.*, 2000), lucifer yellow (Ji *et al.*, 2002), lysotracker (Werneburg *et al.*, 2002) and FITC-dextran < 70kDa (Bidere *et al.*, 2003) can be released. This process has been proposed to be rather limited by size criteria, since FITC-dextran of molecular weight superior to 70kDa were unable to be released from lysosomes of T lymphocytes treated with staurosporin whereas FITC-Dextran of 10 and 40 kDa were redistributed in the cytosol (Bidere *et al.*, 2003). Based on these observations, the release of gentamicin from permeabilized lysosomes is certainly a possible event since its molecular weight is only 464 Da.

However, during our work a retrograde transport of gentamicin has also been demonstrated on LLC-PK1 using similar doses of 1 mg/ml of gentamicin (about 2.15 mM). This transport has been shown to be rapid since gentamicin was already associated to golgi structures after 15 min and to the endoplasmic reticulum after 1 hour of incubation with the antibiotic labelled with Texas-Red fluorophore (Sandoval *et al.*, 2000; Sandoval and Molitoris, 2004). Moreover, after 2 hours of contact with drug, cytosolic release was observed and subsequent nuclear association after 4 hours (Sandoval *et al.*, 2000). However, if we analyze more thoroughly these observations, it may raise some questions. Firstly, the probe used to labelled Golgi structure, NBD-C_6-ceramide demonstrates some limitation. Indeed, NBD-C_6-ceramide can also be inserted in other intracellular membranes (ER, nucleus or mitochondria). Moreover, this probe has been shown to be used for a finite period of time since it is rapidly metabolized by cells (van Meer *et al.*, 1987) and finally its trafficking into cells can be modified by some cellular alterations as it was shown in human skin fibroblasts from patients with Niemann–Pick disease, which is characterized by a lack of lysosomal sphingomyelinase activity, where NBD C_6-ceramide accumulates in the lysosomes. Secondly, the use of gentamicin-labelled with Texas Red can raise the question of the impact of such structural changes on

its trafficking in cells? Indeed, gentamicin is a small cationic compound of a molecular weight of 464 Da. The addition of Texas Red, which is a more lipophilic compound of 625 Da, could indeed modify the general characteristic of gentamicin and thus its trafficking inside the cell. Moreover, in their more recent study, Sandoval and Molitoris were not able to detect any co-localization with the Golgi apparatus by the use of non-fluorescent gentamicin detected by immunolocalization obtained after amplification technique but they observed a reticular staining pattern throughout the entire cytosol from 15 minutes and a co-localization with ER-epitope Dolichos-phosphate mannose synthase after 60 min, cytosolic release was observed after 2 hours followed by a nuclear staining after 4 hours. In addition, all these localizations of gentamicin could also be observed after release from lysosomal destabilization as suggested by our work and more direct proofs are certainly needed to identify clearly hown gentamicin can reach the cellular cytosol. Finally, Sandoval and Molitoris have also tested the cytosolic release of some different probes [a cationic lysine-conjugated dextran (with a molecular weigh of 3,000 Da), a cationic lysine-conjugated dextran (with a molecular weight 10,000 Da) and an anionic derivates dextran (with a molecular weight of 3,000 Da)] to assess the involvement of specific transport from ER to the final cytosolic localization of these endocytozed compounds. Only the small cationic lysine-conjugated dextran has been shown to reach cytosol after 2 hours. This observation is rather interesting, since small cationic compounds have been shown to have destabilizing properties on membrane (Hwang and Vogel, 1998), and thus it could be interesting to analyze the lysosomal stability by using acridine orange probe.

So in conclusion, works carried out by Sandoval and Molitoris clearly demonstrate that gentamicin can be found associated with the ER, in the cytosol and finally localized in the nucleus and mitochondria of LLC-PK1 cells by different morphological approaches whereas Golgi association was observed only by one morphological approaches. They also suggest that aminoglycosides can induce cellular alterations by reaching cytosolic

compartment as also demonstrated by our work. However, the way by which they can really reach cytosol has been proposed to result from lysosomal destabilization by our work (Servais *et al.*, 2005) or by a retrograde transport (Sandoval *et al.*, 2000; Sandoval *et al.*, 1998; Sandoval and Molitoris, 2004). But undoubtedly, the identification of the precise cytosolic release of aminoglycsides is certainly of critical importance to understand their nephrotoxicty.

2.3. What can we conclude about the involvement of lysosomal cathepsins in gentamicin-induced apoptosis?

In our attempts to test the involvement of cathepsins in gentamicin-induced apoptosis, we have first analyzed if addition of cathepsin B and cathepsin D inhibitors, leupeptin and pepstatin A respectively, could rescue cells from gentamicin-induced apoptosis. Surprisingly, the only presence of leupeptin (100 µM) in LLC-PK1 cells was able to induce apoptosis and the presence of this inhibitor plus gentamicin has been shown to induce an increased number of apoptotic cells in an additive fashion. This observation that leupeptin can induce apoptosis was already reported by others who have demonstrated that incubation of primary culture of hepatocytes with leupeptin induced apoptosis (Maeda *et al.*, 1996). But how is this possible? Firstly, leupeptin is a reversible competitive inhibitor of serine and cysteine proteases (Umezawa, 1976) and it has also been shown to inhibit trypsin-like activities (Wilk and Orlowski, 1983; Rivett, 1989). As the proteasome is a multicatalytic complex with trypsin-like activities, the apoptotic ability of leupeptin could indeed be explained by its inhibition properties toward the proteasome and it was indeed shown that leupeptin concentration required to inhibit all the trypsin-like activity of the proteasome is about 50µM (Savory and Rivett, 1993). Secondly, since it is now well established that the inhibition of the proteasome often leads to apoptosis in numerous cell lines (Drexler, 1997; Lopes *et al.*, 1997; Qiu *et al.*, 2000; Drexler *et al.*, 2000; Jana *et al.*, 2001; Powell *et al.*, 2005; Nam *et al.*, 2001; Jana *et al.*,

2004), it is conceivable that leupeptin induces apoptosis in our model due to an inhibition of the proteasome. However, even if in most situations, inhibition of the proteasome results in apoptosis, it has also been demonstrated that, at least in some cases, the proteasome inhibition results in protection of cells from apoptosis (Sadoul *et al.*, 1996).

In conclusion, since we have tested different concentrations of leupeptin up to high concentration of 100µM, we can assume that cathespin B is not involved in gentamicin-induced apoptosis.

What about cathepsin D? Our experiments using pepstatin A have demonstrated that LLC-PK1 cells are protected from gentamicin-induced apoptosis. As cathepsin D has to be located into the cytosol to be a pro-apoptotic mediator (Roberg *et al.*, 2002), we have further analyzed if this protease is released from the lysosomes to the cytosol by cell fractionation. However, any relocation of cathepsin D could be detected in gentamicin-treated cells in comparison to control cells whereas disruption of lysosomes by lysosomal detergent MSDH has allowed us to detect a relocation of cathepsin D from granular fraction to the supernatant.

But how can we explain this discrepancy between pepstatin A studies and fractionation studies? In our pepstatin A experiments, we have previously tested different concentrations of pepstatin A ranging from 50 to 150 µM and we observed that only concentrations of 100 and 150µM of pepstatin A affords protection of cells from gentamicin-induced apoptosis. Such concentration has also been used in other model of apoptosis to test the involvement of cathepsin D (Boya *et al.*, 2003; Kagedal *et al.*, 2001a; Roberg *et al.*, 1999; Isahara *et al.*, 1999), however some other similar studies have been conducted with lower concentrations of the inhibitor (1µM to 50 µM) (Takuma *et al.*, 2003; Bidere *et al.*, 2003; Choi *et al.*, 2002; Kagedal *et al.*, 2001b). In one of these studies, they demonstrate that the addition of 1µM of pepstatin A in J774 inhibits about 80%

of the cathepsin D activity (Kagedal *et al.*, 2001b). As we used high concentration of pepstatin A, it could be possible that other cellular proteases were also inhibited. This was supported by the fact that (1) pepstatin A is not a specific inhibitor of cathepsin D, indeed it has been shown to inhibit acidic proteases among them we can cite pepsin and renin (Marciniszyn 1977 199) and by the fact that (2) non-specific inhibitors can inhibit other proteases in classical used concentration (for exemple: z-VAD-fmk., firstly though to be specific inhibitor of caspases have been shown also to inhibit cysteine proteases (Schotte *et al.*, 1999) and finally by the fact (3) that increasing concentration of a non-specific inhibitors can allow the inhibition of a protease non inhibited in normal conditions (Schotte *et al.*, 1999).

In conclusion, based on our experiments, we can not involve or exclude cathepsin D from gentamicin-induced apoptosis and further analyses are certainly needed to answer this question.

2.4. What is the relevance of the amount defined by electroporation as "apoptogenic" with in vitro observations in LLC-PK1 cells?

By electroporation studies, we have determined extracellular concentration of 0.03 mM as apoptogenic drug concentration and this extracellular concentration has given cellular drug content after 1 hour post-electroporation of 0.32 ±0.03 µg/mg protein and after 24 hours of 0.38 ±0.02 µg/mg protein. After 24 hours, incubated cells with extracellular concentration of gentamicin of 2mM have shown cellular drug content of 3.81 ± 0.06µg/mg protein that is to say cellular drug content ten times superior to that found in electroporated cells. The major difference between electroporation delivery of gentamicin and delivery obtained by incubation is the final drug localization. Indeed, after electroporation the pool of the drug is mainly cytosolic whereas drug localization after incubation is mainly found in lysosomes (Sandoval *et al.*, 1998; Sandoval *et al.*, 2000;

Sandoval and Molitoris, 2004). Using gentamicin labelled with texas red, Sandoval has widely shown that in LLC-PK1 cells incubated with 1mg/ml of gentamicin (2.15 mM), about 80% of the drug localization is the endosomal / lysosomal compartment. Only about 10 to 20% have been associated with golgi, endoplasmic reticulum, mitochondria, nucleus and cytosol. Thus, based on this information, we can postulate that the non-lysosomal amount of gentamicin in incubated LLC-PK1 is about 0.381 to 0.762 µg/mg protein. These contents correspond totally to the drug cellular contents defined by electroporation has being able to induce apoptosis in absence to necrosis (see figure 4 in Servais et al, in press AAC). If we make similar approximation to assess quantity of drug able to induce necrosis, we can calculate (see insert figure 4 in Servais 2006 in press) that cells incubated with gentamicin 4 to 12 mM give an approximate drug content (after 24 hours of incubation) of about 7.4 to 21.9 µg/mg protein and a LDH release after 24 hours of about 29.1% and 58% respectively (See figure 1 in Servais (Servais *et al.*, 2005)). In electroporated cells, the release of LDH was observed for cells which have achieved gentamicin content after 1 hour of 11.3 to 49.8 µg/mg protein with a LDH release observed after 24 hours of 27.9% and 69.5% respectively. When necrosis is analyzed as a cellular response to gentamicin exposure, we observe that the drug cellular content in both cases (electroporated versus incubated cells) are similar suggesting that probably the majority of gentamicin content should be released in the cytosol.

The parameters used to electroporated gentamicin into LLC-PK1 cells (8 pulses – 800V/cm – 1s between each pulses) are parameters allowing the perturbation of plasma membrane without major changes in the other intracellular organites. Indeed, it has been shown that higher voltages (about 300 000 V/cm) for shorter time pulses (in the range of the nanosecond) are needed to electroporated intracellular organites (Schoenbach *et al.*, 2001; Beebe *et al.*, 2003). But, if electroporation technique allows us to analyze the cellular response to gentamicin from cytosolic localization, this technique also

demonstrates some limitations. Electroporation of cells could make them more fragile to gentamicin toxicity. Indeed, this greater susceptibility could result from production of ROS that can be observed after electroporation (Bonnafous *et al.*, 1999) and known to be harmful for cells. This could be tested by electroporating cells with electroporation buffer free of gentamicin and by incubating cells further with gentamicin to compare apoptotic response in these conditions in comparison to “simply” incubated cells, and this could be also tested for another nephrotoxic drug such as cisplatin or cyclosporine A. In addition, we can not exclude that gentamicin can also destabilize lysosomes from the cytosol, this could be analyzed by checking lysosomal stability after electroporation by the use of acridine orange.

2.5. How cytosolic gentamicin can induce cell death?

Different hypotheses can be postulated to explain how gentamicin induces cell death by apoptosis. The first one referred to old suspicions that aminoglycosides can be toxic by interactions with their “first” target, the 18S rRNA (the eukaryotic counterpart of the 16S rRNA). The second one involved the proteasome, which arouse currently an increasing interest as new target for the development of new chemotherapeutic approaches. And finally, the third hypothesis consists of a direct interaction of gentamicin with the mitochondria.

2.5.1. Gentamicin acts on its primary target: 18S rRNA (the eukaryotic homologue of the 16S rRNA):

A. It is conceivable that gentamicin binds to the 18S rRNA?

It was first postulated that aminoglycosides affinity to the eukaryotic 18S rRNA binding site was very low in comparison to the bacterial 16S rRNA due to differences observed in their rRNA sequences (Recht *et al.*, 1999). However, this last five years, the numerous analysis aiming to define the precise binding of the aminoglycosides inside the bacterial 16S rRNA A-site has challenged this

first concept. Indeed, as described in part 1.2.1 of the introduction, it appears now clearly that aminoglycoside binding in the bacterial A-site is firstly dependent of the insertion of this molecule inside a sort of “bubble” created by a non Watson-crick base-pair of the adenines 1408 with both adenines 1492 and 1493 (see figure 27).

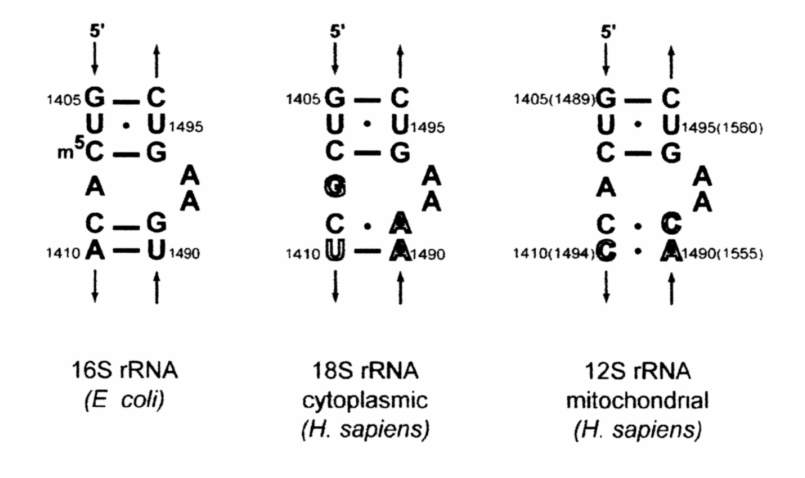


Figure 27: *Sequences of aminoglycosides targets sites rRNA species. In every site, the two adenines 1492 and 1493 (according to the E. coli numbering) are conserved. Mutation of the adenine at position 1490 according E. coli numbering and 1555 according H. sapiens numbering of the human mitochondrial 12S rRNA to guanine is associated with Aminoglycosides hypersensitivity.This figure come from Vicens and Westhof (Vicens and Westhof, 2003b)*

This concept of aminoglycoside insertion in a sort of bubble created in the RNA was indeed supported by the fact they bind to small ribosomal subunit of many other prokaryotic and eukaryotic species presenting such structural characteristics, but also by the demonstration that they bind to mutated human mitochondrial 12S rRNA whereas they can not bind to the wild-type 12S rRNA (see figure 27) (Ryu and Rando, 2001; Hamasaki and Rando, 1997; Ryu and Rando, 2002). This mutation results in the replacement of the adenine in position 1490 (according *E. coli* numbering) by a guanine restoring the Watson-Crick link, thus reducing the size of the rRNA bubble which presents then a similar size to that observed in the 18S rRNA (Ryu and Rando, 2002). Moreover, this mutation of the 12S rRNA observed in certain individuals has

been associated with aminoglycoside hypersensitivity (Hutchin and Cortopassi, 1994) since treatment these individuals with normal, low doses of aminoglycosides, results in toxic side effects on hair cells of the inner ear that may end in permanent hearing loss. So, based on this “new view” of aminoglycoside insertion, it seems conceivable that they can interact with the 18S rRNA, if of course they have access to the ribosome. But is it possible that aminoglycosides have access to the ribosomes?

If differences of RNA sequences between prokaryotic 16S and 18S rRNA have been postulated to explain the dissociation between antibacterial activity and toxicity, another argument had also been proposed which consist of a non accessibility of the drug to the ribosomes. Indeed, the interaction of aminoglycosides with the human ribosome was first thought to be minor and probably insignificant since the bulk of the drug localization in kidney cells was for a long time described to be exclusively inside the lysosomes and thus far away from their ribosomal target (Houghton *et al.*, 1976; Silverblatt and Kuehn, 1979; Wedeen *et al.*, 1983; Ford *et al.*, 1994). However, some intriguing studies have yet demonstrated that elevated concentration of aminoglycosides can cause mistranslation or block incorporation of amino acids by eukaryotic ribosomes *in vitro* (Buss *et al.*, 1984; Buss and Piatt, 1985; Wilhelm *et al.*, 1978), but moreover this was also observed in the rat renal cortex during *in vivo* treatment (Buss *et al.*, 1984; Buss and Piatt, 1985; Bennett *et al.*, 1988). The ability of gentamicin to inhibit protein synthesis has been more recently also observed in rats treated for 2 days of treatment with gentamicin (40mg/ml) (Sundin *et al.*, 2001) but also in LLC-PK1 cells incubated with gentamicin 1mg/ml (2.15 mM) (Sandoval *et al.*, 1998) before clear sign of nephrotoxicity can be detected. So there nearly no doubt that gentamicin can perturb protein synthesis in clinical conditions, the only remaining question is if gentamicin can access to ribosome. The answer has been given by the localization studies performed by Molitoris and Sandoval group, who have demonstrated that indeed, using clinical relevant doses, gentamicin have been found localized in

small quantity with the Golgi, the endoplasmic reticulum, the mitochondria and nucleus (Sandoval *et al.*, 2000; Sandoval and Molitoris, 2004) resulting from a retrograde transport through the secretory pathway. Furthermore, our observations, that it is the cytosolic gentamicin which induce apoptotic death pathway have also reinforced this possibility.

So, in conclusion, what ever the origin of aminoglycosides, from a retrograde transport as proposed by Molitoris and Sandoval group, or from a lysosomal destabilization as proposed by our experiments or both, gentamicin can access cytosolic compartment and thus are able to reach their "first" target, the ribosomal 18S RNA A-Site.

B. But how can we now link the aminoglycosides effect of on their rRNA target (18S rRNA) with apoptosis? What about ER stress?

On one hand, gentamicin has been shown to perturb and finally inhibit protein synthesis (Buss *et al.*, 1984; Buss and Piatt, 1985; Bennett *et al.*, 1988; Sandoval *et al.*, 2000; Sundin *et al.*, 2001). The perturbation of protein synthesis has also been shown to be induced by different toxins and components regrouped and named the RIP, for Ribosomal Inactivating Protein, and among these components, some of them such as verocytotoxin-1 (Barbieri *et al.*, 1993), ricin, modeccin, diphtheria toxin (Komatsu *et al.*, 1998) have been shown to induce apoptosis. However, at the present, the molecular link between the production of abnormal protein and/or protein synthesis inhibition and triggering of apoptotic cell death is unclear and it remains now numerous unresolved questions.

On the other hand, considering that the ER is constantly monitoring processing via the quality control machinery what is the response if this process is compromised, for exemple by the accumulation of misfolded/unfolded proteins within the ER (as induced by gentamicin action on the ribosome)? In reaction to

these conditions, the cell trigger a specific ER stress response (Rutkowski and Kaufman, 2004) that activates a particular ER-stress signalling pathway termed Unfolded Protein Response (UPR). This pathway is a cellular program which is activated by a complex signalling cascade and involves at least three different mechanisms to re-establish homeostasis including the attenuation of protein synthesis (Brostrom *et al.*, 1996; Harding *et al.*, 1999), the activation of UPR targets gene increasing for exemple the production of chaperones such as Grp78 (also called BiP), Grp 94 or Protein Disulfide Isomerase (PDI) (Gething and Sambrook, 1992), as well as the enhancement of abnormal protein degradation (Jeffery *et al.*, 2000). This degradation is accomplished by a specific system termed ERAD for ER-Associated Degradation responsible for the unfolding and elimination of the misfolded proteins by targeting them to the cytoplasm to be degraded by the 26S proteasome (Tsai *et al.*, 2002; Hirsch *et al.*, 2004). If at some point the cell is overwhelmed with unfolded protein and is unable to recover, the feedback mechanism facilitates a signal to the apoptotic pathway from the ER (see introduction) (Yoneda *et al.*, 2001; Zuppini *et al.*, 2002).

So if gentamicin accesses the ribosome resulting in production of misfolded proteins, it is possible that this event will induce stress in the ER. This possibility was indeed supported by the fact that a parent compound to gentamicin, the G418 (a neomycin analogue, well known for its potent protein synthesis inhibition in eukaryotes by binding the 80S ribosome (Bar-Nun *et al.*, 1983)) have been show to induce apoptosis (Billaut-Mulot *et al.*, 1996) which was associated to caspase activation resulting from ER stress and cytochrome c release (Jin *et al.*, 2004). Moreover, the possible induction of ER stress by gentamicin is also supported by the observations that gentamicin can binds and inhibits the chaperone activity of calreticulin (Horibe *et al.*, 2004). This protein functions as a lectin-like chaperone and works as part of the ER quality control that couples the processing of N-linked glycans with protein folding (Krause and Michalak, 1997). Furthemore, calreticulin is not the only-chaperone that

gentamicin can bind, the other ER protein, Protein Disulfide Isomerase (PDI) has also be found to bind gentamicin (Horibe *et al.*, 2001; Horibe *et al.*, 2002). Taking together all these elements, gentamicin could be a powerful ER-stressor since it can not only produce misfolded proteins and thus induce ER stress but it can also interfere with chaperones involved in the recovery cellular activated after mild ER stress.

In our studies, we have also considered the involvement of ER pathway in gentamicin-induced apoptosis. In a first approach, we have performed preliminary morphological analyses by electronic microscopy of LLC-PK1 cells treated with gentamicin 3 mM for 48 hours and have observed an ER dilatation (see figure 28) which was not observed in control cells (data not shown). This observation should be analyzed more precisely to assure that this is significant in comparison to control and should be tested for shorter time of incubation but also lower concentration of gentamicin.

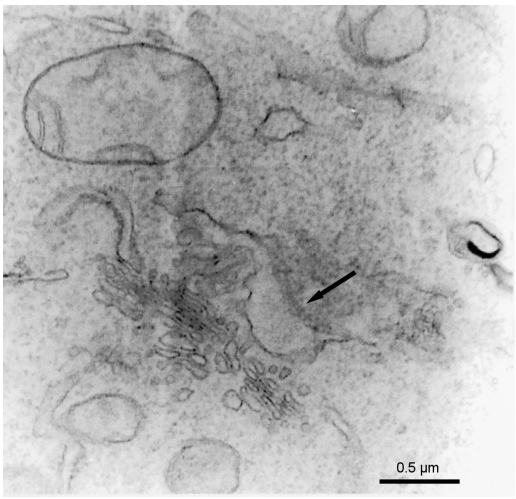


Figure 28: *Electronic micrograph showing a dilatation of the ER in LLC-PK1 cells treated 48 hours with 3 mM of gentamicin*

Based on these observations, we further analyzed if caspase-12, at that time described as ER specific caspase, was activated in gentamicin-induced apoptosis by western blot analysis and did not found any detection. This result was now more easily interpreted since caspase-12, previously described as ER-

specific (Nakagawa *et al.*, 2000), was not detected in most humans (Fischer *et al.*, 2002). As our cellular model consist of kidney cell originating from pig it is probable that this caspase is also absent. Caspase-4 has been proposed to be ER-stress specific but controversies remain about this issue. Identification of the functional homologue of caspase-12 in humans and its physiologic significance in ER-stress induced apoptosis is an important issue yet to be addressed.

2.5.2. **Gentamicin could act by altering the 26S proteasome.**

The hypothesis that proteasomal inhibition can be induced by gentamicin in a direct or indirect way can be supported by our results demonstrating an increase of ubiquitinated Bax. Indeed, the accumulation of ubiquitinated proteins has been often associated with proteasome dysfunctions (Jana *et al.*, 2004; Kazi *et al.*, 2003; Kuhn *et al.*, 2004; Powell *et al.*, 2005). The pro-apoptotic Bax protein has been shown to be degraded in cells via the proteasome pathway (Li and Dou, 2000), and it has been shown that the addition of proteasome inhibitors in Jurkat T cells induces an increase of Bax but also from its ubiquitinated form. This was observed before mitochondrial release of cytochrome c into the cytosol and activation of caspase-mediated apoptosis (Li and Dou, 2000). In the gentamicin-induced apoptosis, we observe also an increase of ubiquitinated Bax but also of its non ubiquitinated form. This pattern of accumulation of both form of Bax has also been observed following incubation of cardiomyocytes with lipofuscin which induce the formation of oxidized proteins aggregates which inhibit the proteasome (Powell *et al.*, 2005). The similar accumulation of both form of a protein (ubiquitinated and non-ubiquitinated ones) seemed not to be protein specific since it is also observed for other proteins such as p27 (Powell *et al.*, 2005; Nakanishi *et al.*, 2000) but also for destabilized enhanced green fluorescent protein (d1EGFP), a model substrate for proteasome with 1-hour half-life (Jana *et al.*, 2004). This suggests

that it should have a balance between both forms of proteins probably resulting from the involvement of deubiquitinating enzymes.

But how can we explain the possible proteasome inhibition in gentamicin-induced apoptosis? (1) As described in the precedent section, Horibe and colleagues have recently shown that gentamicin can bind to some ER chaperones but they also observed that it bind to another protein identified as PSB 9 (proteasome subunit β type-9) (Horibe *et al.*, 2004). PSB9 consists of the precursor of the proteasome subunit β type-7 which functions to amplify specific endopeptidase activities of the proteasome and contribute preferentially to the hydrolysis of hydrophobic and basic substrates (Van Kaer *et al.*, 1994). So, we can suspect that gentamicin interfere with the proteasomal function. (2) However, we can not exclude that the alteration of the proteasome activity is induced by the accumulation of misfolded/unfolded proteins. Indeed, in several pathologies associated with production of abnormal proteins such as mutant Huntigton (Bence *et al.*, 2001; Jana *et al.*, 2001), the aberrant ubiquitin UBB^{+1} (Lam *et al.*, 2000; Lindsten *et al.*, 2002) have been shown to negatively affect the UPS supporting a role of the proteasome dysfunction in these conformational diseases. Moreover, impairement of the proteasome as a consequence of these proteins is indeed usually followed by rapid cell death by apoptosis (Bence *et al.*, 2001; Lindsten *et al.*, 2002; de Vrij *et al.*, 2001). (3) It has been recently demonstrated by Menendez-Benito and colleagues, which have shown that ER stress have a general inhibitory effect on the proteasome (Menendez-Benito *et al.*, 2005) but the molecular link between both of these events are not known. (4) And finally, generation ROS has also be associated with proteasome dysfunction (Ding and Keller, 2001; Okada *et al.*, 1999). In conclusion, both of these four hypotheses are plausible, since gentamicin has been show (1) to bind to a precursor of the proteasome (Horibe *et al.*, 2004), (2) could produce abnormal proteins by interaction with the eukaryotic ribosomes (Ryu and Rando, 2001), (3) could induce ER stress (Horibe *et al.*, 2002; Horibe

et al., 2004; Jin *et al.*, 2004) and (4) produce ROS generation (van der Harst *et al.*, 2005; Walker *et al.*, 1999; Guidet and Shah, 1989).

2.5.3. Gentamicin could directly disturb mitochondria

In the first part of our work, we have firstly demonstrated that mitochondrial potential was disturbed (after 10 hours of contact with the drug) and later that cytochrome c was released and caspase-9 activated after 12 hours of incubation with 2 mM of gentamicin. Thus, mitochondria are involved in gentamicin-induced apoptosis. In addition, we have analyzed the destabilization ability of gentamicin on liposomes of composition mimicking lysosomal membrane, inner- and outer-membrane of mitochondria. Our results showed that gentamicin destabilized all type of liposomes with decreasing rapidity, suggesting that gentamicin could activate mitochondria by a direct interaction. This hypothesis was supported also by the work of Mather and Rottenberg, which have analyzed the effect of three aminoglycosides, neomycin, gentamicin and streptomycin on the release of cytochrome c from isolated mitochondria and if this release relies upon Permeability Transition Pore (PTP) opening. The release of cytochrome c from mitochondria by aminoglycosides was inhibited by cyclosporine (an inhibitor of the PTP pores) to various extents, depending on the antibiotic used. Streptomycin-induced cytochrome c release was almost completely inhibited by cyclosporine, gentamicin-induced cytochrome c release was partially inhibited while neomycin-induced cytochrome c release was only slighty inhibited (Mather and Rottenberg, 2001). These results suggested that aminoglycosides can induce the release of cytochrome c by directly destabilizing the outer membrane but also by inducing PTP opening. The ability of molecules that contain clusters of positive charges (as it is the case for aminoglycosides) to disturb membrane has indeed been demonstrated for several cytotoxic peptides produced by insects, bacteria and other organisms (Hwang and Vogel, 1998) and some of these compounds have also been able to induce cytochrome c release and subsequent apoptosis (Ellerby *et al.*, 1999;

Stefanelli *et al.*, 1999). More interestingly, pro-apoptotic member of the Bcl-2 family and in particular Bax carries a cluster of positively charged residues in its membrane-insertion domain and this protein has also been shown to destabilize not only liposomes (Kuwana *et al.*, 2002) but also the cellular mitochondria by its insertion in this organelle (Antonsson *et al.*, 1997) and/or by interaction with the PTP (Marzo *et al.*, 1998; Shimizu *et al.*, 1999).

The action of gentamicin on mitochondria is also suspected to be responsible for the shift of cell response to gentamicin from an apoptotic one to a necrotic one. Indeed, it has been proposed that the induction of mitochondrial permeabilization in a small fraction of mitochondria could activate the apoptotic process, whereas induction of mitochondrial permeabilization in a large portion of mitochondria could cause bioenergetic collapse and thus ATP depletion, leading to necrosis. In the case of gentamicin, this could explain the change of response observed in LLC-PK1 cells treated with increasing concentration. From 1 to 3 mM, gentamicin induces apoptosis whereas higher concentrations (4 to 12 mM) induce necrosis. This change of cellular response towards gentamicin insult has also been observed in electroporated cells suggesting that reaching a certain cellular concentration, the insult to cell is too high. This change of response could be attributed to more massive lysosomal destabilization with the release of high amount of gentamicin.

2.6. What about the evolution of our comprehension of aminoglycosides nephrotoxicity?

As mentioned earlier, aminoglycosides are clinical important antibiotics due to their efficacy towards severe and often life-threatening infections caused by Gram negative bacteria. However, their use is often limited by their toxic potential in the kidney and in the inner ear. Even if nephrotoxicity of the aminoglycosides has been widely studied these last decades, the exact mechanism underlying their toxicity was not clearly identified. Numerous alterations have indeed been associated to aminoglycosides, and some of them

arousing controversies. The critical advances made this last ten years on the aminoglycosides domain may certainly allow the pieces of the puzzle to fall into place. In this section, we will illustrate which is the evolution of our understanding of the aminoglycosides nephrotoxicity.

1980-1999: Lysosomal phospholipidosis is a key event in aminoglycosides nephrotoxicity.

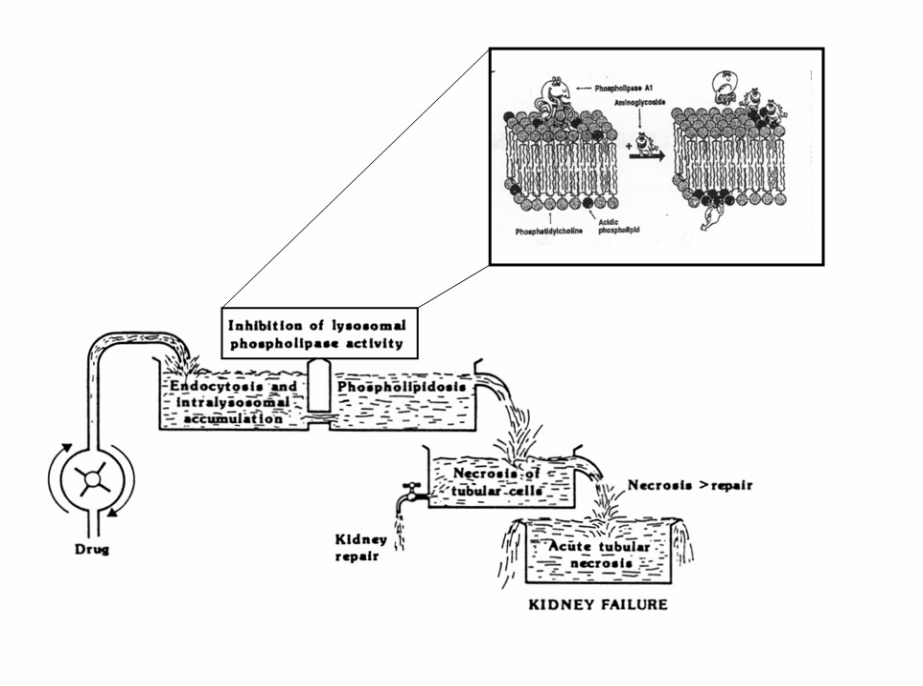


Figure 29: *Proposed sequence of pathological events occuring in kidney proximal tubular cells after administration of aminoglycosides at low doses. Note that the relationship between phospholipidosis and necrosis is not proven.Phospholipidosis had been attributed to the lysosomal phospholipase and sphingomyelinases inhibition (see insert) (Laurent et al., 1982; Hostetler and Hall, 1982). The pump reflects the fact that the endocytic uptake of aminoglycosides in lysosomes results in a considerable (100-1000 fold) accumulation of these drugs over serum ultrafiltrate concentration, and also that this uptake is dose-saturable. The different levels of containers indicate that the "downstream" phenomenon starts only when the process occurring "upstream" reaches a critical threshold level. In rats treated with low, therapeutic doses of aminoglycosides, focal tubular necrosis is compensated for by repair, and no kidney dysfunction is observed. A major reason for the greater risk of humans, especially old patients, than of animals may be that the regenerating capabilities are lower or impaired. This illustration has been adaptated from Laurent and Tulkens (1987). (Laurent and Tulkens, 1987) Insert has been adapted from Mingeot-Leclercq (Mingeot-Leclercq M.P. et al., 1991).*

The proposed toxic mechanism of aminoglycosides at that moment included: (1) the uptake of these antibiotics by proximal tubular cells, where they were first sequestered within the lysosomes and (2) the development of a lysosomal phospholipidosis, which had been rapidly associated with cell necrosis and various alterations to subcellular structure and function. Phospholipidosis had been shown to be attributed to the inhibition of lysosomal phospholipases and sphingomyelinases (Laurent *et al.*, 1982). Tubular necrosis is often accompanied by (and probably triggers) tubular regeneration and peritubular proliferation. The means whereby such tubular alterations eventually caused a decline in glomerular filtration and hypo-osmotic polyuria had not been established. Various in vitro and acellular models had been designed to assess and screen for the nephrotoxic potential of aminoglycosides; of these, method based on the analysis of aminoglycosides-phospholipid interactions appeared to be the more meaningful since interaction between aminoglycosides and membranes and/or enzymes associated to membranes had been often pointed out. Aminoglycosides indeed neutralize negatively charged liposomes (Alexander *et al.*, 1979; Chung *et al.*, 1985), they displace calcium from negatively charged phospholipids monolayers and perturb their surface tension (Au *et al.*, 1986), but also the phospholipidosis has been explained by the interaction of aminoglycosides with acidic phospholipids necessary to phospholipase activity (Mingeot-Leclercq *et al.*, 1990a; Mingeot-Leclercq *et al.*, 1990b).

1999-2000: Apoptosis is a key event in aminoglycosides nephrotoxicity.

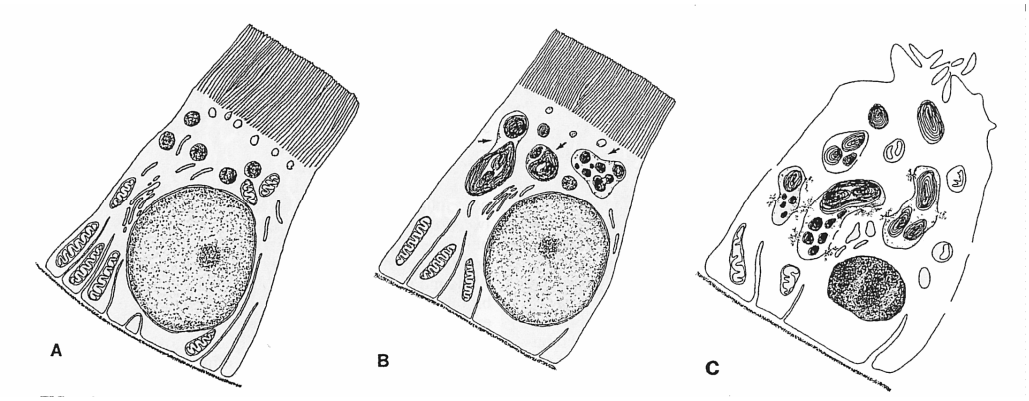


Figure 30: *Ultrastructural alterations induced in proximal tubular cells during aminoglycosides treatment. (A) Control. Changes detected early on and at low doses (B) consist mainly of the enlargement of the lysosomes, whoch most likely occurs by fusion of pre-existing structures and which is caused by the progressive deposition of polar lipids which adopt a concentric lamellar disposition (myelin-like structures, most commonly referred to as myeloid bodies); the other subcellular structure are usually well preserved. Later changes or changes observed at high doses (C) include the apparent rupture of lysosomes (with the release of myeloid bodies in the cytosol), extensive mitochondrial swellingand damage, dilatation of the endoplasmic reticulum cisternae, shedding of theapical brush-border villi, pericellular membrane discontinuities, and the occurrence of apoptotic nuclei. These alterations do not necesseraly coexist in all cells. This figure originated from Mingeot and Tulkens (Mingeot-Leclercq and Tulkens, 1999).*

The observations that aminoglycosides were able to induce apoptosis, *in vivo* and *in vitro* at doses relevant to clinical situations, had shed new lights on the early aspects of their toxic mechanisms (El Mouedden *et al.*, 2000b; El Mouedden *et al.*, 2000a). Apoptosis had been shown not only to be associated with nephrotoxicity (gentamicin the most nephrotoxic compound studies by El Mouedden and colleagues had demonstrated to be the greater inducer of apoptosis) but also to be function of the time of exposure and of the drug concentrations. In addition, apoptosis-induced by these antibiotics had been correlated with the cellular drug content. However, phospholipidosis was not found unambiguously correlated to apoptosis.

2006: Cytosolic gentamicin is responsible for the induction of apoptosis involving the mitochondrial pathway and the activation of caspases. The sequestration of gentamicin in lysosomes could, to some extent, protect cells from apoptotic and necrotic cell death.

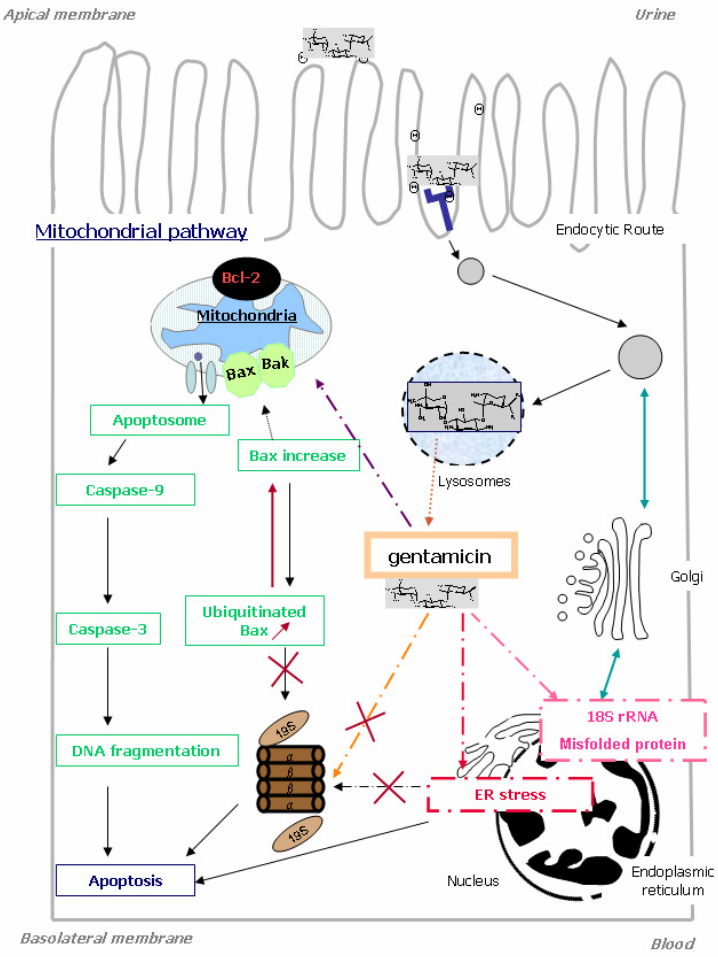
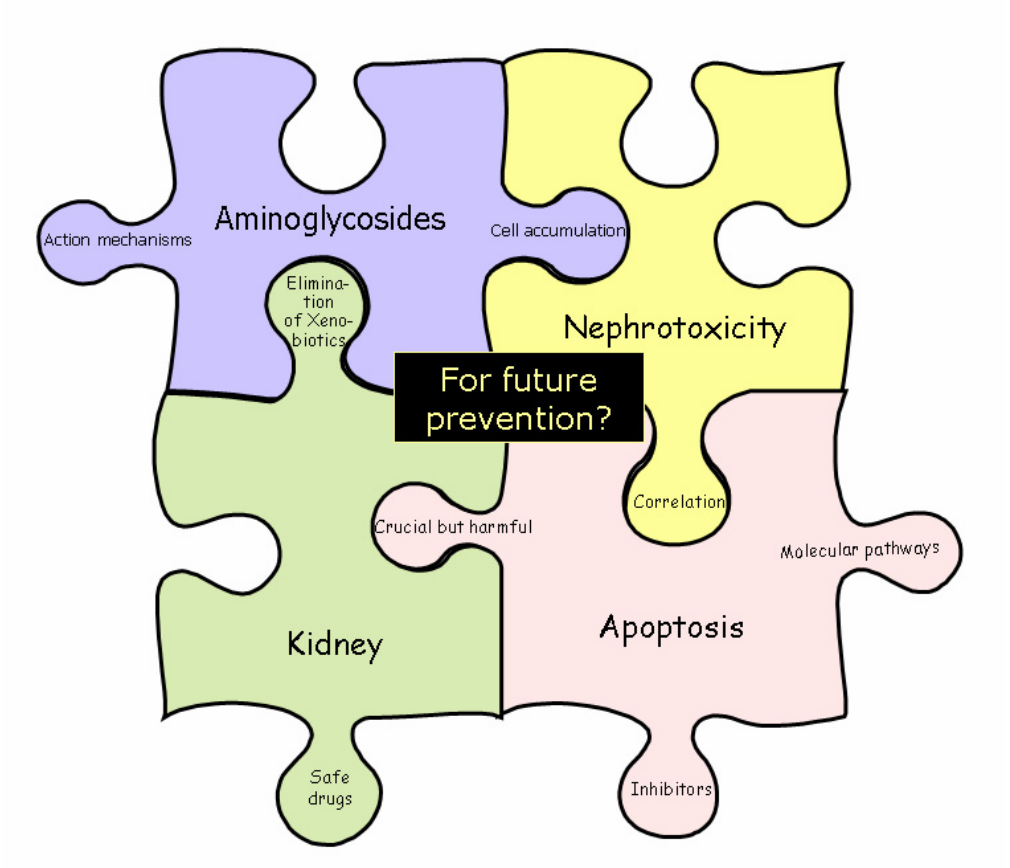


Figure 31: *Apoptosis-related changes observed in renal cells. The induction of apoptosisoccurs when gentamicin reaches the cytosol either after its release from the destabilized lysosomes as proposed by our work (Servais et al., 2005) and/or by a retrograde transport as proposed by Molitoris group (Sandoval and Molitoris, 2004). The sequestration of the aminoglycosides inside the lysosomes, could rather, in some extent protect cell from apoptogenic gentamicin. Apotosis induced by gentamicin involved the mitochondrial pathway and Bax and the activation of caspase-9 and caspase-3. The way by which cytosolic gentamicin induces mitochondrial and caspase activation is not known but it could involved (1) an alteration of protein synthesis, (2) an ER-stress, (3) the inhibition of the proteasome or finally a direct interaction of gentamicin with mitochondria. Both of these events have been shown to activate apoptosis.*

The observation that cytosolic gentamicin can induce apoptosis at only low concentration has brought new elements to explain how this antibiotic induce cell death and thus nephrotoxicity. Surprinsingly, this also challenged our first thinking that aminoglycoside-induced cell death should be linked to lysosomal alterations and defining lysosomes as ***weapons***. However, the lysosomes could be rather a protection system against harmful aminoglycosides and thus defining them rather as the cell ***shield***.

Chapitre V: PERSPECTIVES



1. SHORT TERM PERSPECTIVES.

1.1. Importance of the lysosomal destabilization

Our observations that cytosolic gentamicin induces apoptosis has challenged our first hypothesis involving lysosomal pathway in gentamicin-induced apoptosis. However, we can not exclude that this event can contribute to the cell death by apoptosis and some question remained unresolved. In this context, it should be interesting to identify which is the mechanism responsible for the lysosomal destabilization, but also to analyze precisely in gentamicin-treated cells which are the components released from destabilized lysosomes and if they contribute to cell death.

To explain the lysosomal destabilization, different hypotheses have been proposed and this could result from: (1) ROS generation due to the formation of a very oxidant Fe(II)-gentamicin complex (Priuska and Schacht, 1997), (2) the direct interaction of the drug with the lipids (Servais *et al.*, 2005), (3) the interaction of the drug with the chaperones hsp73 involved in lysosomal functions and stability (Miyazaki *et al.*, 2004; Nylandsted *et al.*, 2004) or finally (4) the overload of these organites by the drug and/or phospholipids accumulation (Kishore *et al.*, 1990).

The lysosomal constituents released from permeabilized lysosomes could be the cathepsins, indeed our experiments have not allowed us to conclude if they are or not released and involved in gentamicin-induced apoptosis, so further experiments are needed to answer this question. But besides the cathepsins, other constituents could also be released such as lactoferrin (Katunuma *et al.*, 2004) since lysosomal permeabilization seemed not to be specific to one lysosomal constituent but rather dependent of its size (inferior to 70kDa, Bidere 2003).

In this context, another challenging question is the definition of the exact origin of the cytosolic gentamicin. This could indeed results from the lysosomal

destabilization (Servais *et al.*, 2005) but also from a retrograde transport through the secretory pathway (Sandoval and Molitoris, 2004). As the delivery of gentamicin in the cytosol is the critical event in the induction of apotosis, it is certainly of great importance to know how gentamicin can reach this localization. However, as both of these pathways are linked to the endocytosis of gentamicin, this is probably a challenging issue.

1.2. Identification of the cytosolic target of gentamicin responsible for apoptosis induction.

1.2.1. Production of misfolded protein and inhibition of protein synthesis by gentamicin.

A. Importance of the interaction between aminoglycosides and the 18S rRNA

The recent observations that aminoglycosides can also bind to the eukaryotic ribosome (Ryu and Rando, 2001) but also the observations that low amount of gentamicin can be localized inside the cellular cytosol (Sandoval and Molitoris, 2004) and able to induce apoptosis (Servais 2006, Antimicrobial Agents and Chemotherapy, *in press*) suggest that aminoglycoside toxicity could be associated with the activity of these antibiotics on the ribosomes. In this context, it could be very interesting to analyze if protein synthesis is observed before apoptosis completion in cells treated with gentamicin, and to test if the different aminoglycosides have different ability to disturb protein synthesis in renal cells and if this occurs in relation to their nephrotoxicity.

As increasing number of compounds inhibiting protein synthesis have been show to induce apoptosis (Williams *et al.*, 1997; Shifrin and Anderson, 1999; Shaw *et al.*, 2005) it could be very interesting to compare the mechanism by which they are able to induce apoptosis. Indeed, a specific pathway could be initiated after protein synthesis inhibition involving some specialized cellular stress sensors.

B. Importance of the ER stress in gentamicin-induced apoptosis

Several studies have yet suggested that ER stress can be induced by gentamicin. Indeed, Horibe and colleagues have demonstrated that gentamicin can binds and inhibit the chaperone activity of two proteins involved in the ER quality control: calreticulin (Horibe *et al.*, 2004) and protein disulfide isomerase (PDI) (Horibe *et al.*, 2001; Horibe *et al.*, 2002). This hypothesis is further supported by the observation that G418, a neomycin analogue, is able to induce apoptosis involving an ER stress pathway (Jin *et al.*, 2004).In addition, the fact that gentamicin can be found in the cytosol and thus able to interact with the ribosome, inducing then the production of misfolded proteins reinforced this hypothesis since misfolded or unfolded proteins is a well-defined inducer of ER stress. In view to all these arguments, the ER stress-pathway leading to apoptosis is a certainly important hypothesis to be checked.

As the ER pathway of apoptosis is now more detailed, its involvement in gentamicin-induced apoptosis could be tested by the measure of the involvement of induced proteins such as CHOP (GADD 153).

1.2.2. **Importance of the inhibition of the proteasome in gentamicin-induced apoptosis:**

Our experiments involving Bax and ubiquitinated Bax suggests that the proteasome could be inhibited in gentamicin treated cells. However, we have no direct demonstration of this phenomenon and it could be thus of importance to check if this occurs in our model of apoptosis. This can be tested by the use of specific substrate of the proteasome determining the different activity of this complex: the trypsin, chymotrypsin, and post-glutamyl peptidyl hydrolytic-like activities (Jana *et al.*, 2004).

As discussed in the precedent chapter, different hypotheses have been raised to explain the proteasome dysfunction: it could result (1) from a direct

interaction of gentamicin with the precursor PSB9 (Horibe *et al.*, 2004), (2) from the production by gentamicin of misfolded proteins overwhelming the proteasome capacity to eliminate these abnormal proteins, (3) from an activation of an ER-Proteasomal pathways (Menendez-Benito *et al.*, 2005) or finally from (4) ROS generation (Ding and Keller, 2001; Okada *et al.*, 1999).

As ER stress have been recently associated with proteasomal dysfunction, it could be interesting to test if this is observed in gentamicin-induced apoptosis or at the opposite, if the inhibition of the proteasome result from a direct action of gentamicin with the proteasome.

1.2.3. Importance of mitochondrial targeting of gentamicin in the induction of apoptosis.

In our first work, we have demonstrated that gentamicin induced an activation of the mitochondria accompanied by the release of cytochrome c and activation of caspase-9 (Servais *et al.*, 2005). As mitochondria seemed to be the central organelle that “decide” if cell will die or not by apoptosis, its involvement in gentamicin is certainly of great importance. This has been indeed supported by the observations of El Mouedden and colleagues showing that the overexpression of Bcl-2 protects cells from apoptosis induced by gentamicin (El Mouedden *et al.*, 2000b). However, at this stage, we do not know which signal is responsible for mitochondrial pathway activation: different possibilities have been proposed such as the activation of Bax (Servais 2006) but also a direct interaction of gentamicin (Servais *et al.*, 2005; Mather and Rottenberg, 2001).

In conclusion, the identification of the cytosolic target of aminoglycosides would certainly help to a better understanding of the mechanisms underlying aminoglycosides-induced apoptosis and the characterization of new approaches to reduce their nephrotoxicity.

2. LONG TERM PERSPECTIVES:

The research on aminoglycosides activity and nephrotoxicity has been abundant this last decade. In particular, the identification of the binding site and the type of interaction of the aminoglycosides with its ribosomal target, the A-site has revived the race towards the development of new aminoglycosides. Yet different university groups or industries has obtained patent for some new "aminoglycosides" (ISIS PHARM.: US6541456 (2003), ISIS PHARM.: US6759523 (2004), OPTIMER PHARM.: US20040058880 (2004), THE SCRIPPS RES.INST.: WO0180863 (2001) or have published promising new molecules and approaches (Ding *et al.*, 2001; Ding *et al.*, 2003; Yao *et al.*, 2004; Vourloumis *et al.*, 2002; Simonsen *et al.*, 2002; Simonsen *et al.*, 2003; Barluenga *et al.*, 2004; Fridman *et al.*, 2003; Chou *et al.*, 2004).

So, in view of all these new compounds, the identification of the exact events leading to toxicity is of crucial importance. Based on all the studies concerning the aminoglycosides nephrotoxicity, we can postulate now that their nephrotoxicity depends on (1) their **capture by the kidney cells**, (2) by their ability to **induce apoptosis** and (3) their ability to **reach cellular cytosol**.

(1) The mechanism by which aminoglycosides are reabsorbed by kidney cells is now well established and occurs by receptor-mediated endocytosis through megalin binding. Indeed, mice with genetic or functional deficiency of megalin have been shown to be unable to capture aminoglycosides in the proximal tubular cells but moreover they were protected towards aminoglycosides nephrotoxicity (Schmitz *et al.*, 2002). The electrostatical interaction of aminoglycosides with the negatively-charged phospholipids of the brush border is also an important event in the aminoglycosides accumulation in proximal tubular cells. This event was indeed yet suggested to be crucial for nephrotoxicity in the late 1980s by Williams and colleagues which had observed a correlation between the renal membrane binding and nephrotoxicity of

aminoglycosides (Williams *et al.*, 1987). This capture of aminoglycosides and analogues could be yet easily tested by the measure of their capture in kidney cells.

(2) In 2000, El Mouedden has brought a new important element contributing in the development of nephrotoxicity by the demonstration that these antibiotics induced cell death by apoptosis (El Mouedden *et al.*, 2000b; El Mouedden *et al.*, 2000a). They have indeed associated nephrotoxicity with apoptosis. In this context, to be able to develop appropriate tests assessing their pro-apoptotic ability, it is certainly critical to know exactly how they induce apoptosis and which is the “inducing” event. In addition, by our experiments of electroporation, we have demonstrated that it is the cytosolic drug which induces apoptosis and that only few amounts of drugs is sufficient to do this. This electroporation approach could be another manner to evaluate the intrinsic potential of new molecules to induce apoptosis requiring only low amounts of drugs.

(3) Finally, the identification of the pathway(s) by which aminoglycosides reach the cytosolic compartment is also of crucial importance since it is from this localization that they induce cell death. Are they differences in the lysosomal destabilization between the different aminoglycosides or are they able to traffic differently through the retrograde secretory pathway are unresolved but important questions. Thus, this third element involving cytosolic access of the aminoglycosides should certainly be taken into account for an appropriate evaluation of the aminoglycosides nephrotoxicity.

So in conclusion, it seems now important to validate such tests (binding to megalin and accumulation in cells, apoptosis-induction, and accessibility to the cytosol) with the existing molecules in order to be able to assess appropriately the nephrotoxicity of the new “aminoglycosides”. Indeed, even if a reduction of toxic side effects have been obtained by ameliorating drug administration

regimen (Once-daily schedule) and by monitoring drug levels, the aminoglycoside toxicity remains a limiting factor to their clinical use. In addition, the monitoring is time-consuming and cost effective in an era where drug use optimization is a real objective.

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RESUME

La gentamicine est un antibiotique de la classe des aminoglycosides, ils sont utilisés en milieu hospitalier pour traiter des infections sévères causées par des bactéries Gram-négatif, cependant leur utilisation est limitée de par la néphrotoxicité qu'ils induisent. Ces antibiotiques sont effet éliminés par filtration glomérulaire et réabsorbés par endocytose recepteur-médiée par les cellules du tube contourné proximal du rein. Ils s'accumulent ensuite de manière importante au sein des lysosomes de ces cellules. La démonstration que ces antibiotiques sont capables d'induire une perte cellulaire par apoptose et cela à des doses correspondantes aux doses cliniques a permis de nouvelles perspectives quant à l'identification des mécanismes responsables de leur toxicité.

Lorsque des cellules rénales, LLC-PK1 (Lilly Laboratory Cell Pig Kidney type 1) sont incubées en présence de gentamicine (1 à 3 mM, de 0 à 72 heures), l'apoptose se développe en fonction du temps et de la concentration extracellulaire en gentamicine. La première altération détectée est une destabilisation des lysosomes dès 2 heures de contact avec la gentamicine, suivie par l'augmentation d'une protéine cytosolique, Bax connue pour induire l'activation des mitochondries, celle-ci (mesurée par la chute de potentiel de membrane mitochondrial) est en effet observée dès 10 heures de contact. Les mitochondries activées permettent ensuite le relargage de cytochrome c après 12 heures de contact avec l'antibiotique, responsable de l'activation de la caspase-9 observée après 12 heures. Cette caspase initiatrice va ensuite activée la caspase-3 (caspase exécutrice et responsable de la plupart des modifications observées lors de l'apoptose des cellules) dont l'activité est détectée après 24 heures en parallèle à la fragmentation nucléaire, caractéristique morphologique des cellules apoptotiques.

La fragilisation des lysosomes engendre un relargage dans le cytosol de composantes pro-apoptotiques, telles les cathepsines B et D mais aussi probablement de la gentamicine elle-même. L'implication de la gentamicine a été étudiée en electroporant les cellules LLC-PK1 afin de délivrer la gentamicine directement dans le cytosol. En effet, dans ces conditions la localisation cellulaire de la gentamicine est majoritairement cytosolique contrairement à sa localisation après incubation qui elle est majoritairement lysosomiale. L'électroporation de quantité très faible en gentamicine (environs 100 fois inférieure à celles requises après incubation) induit de l'apoptose alors que l'électroporation des doses plus importantes (correspondantes à celles requises pour induire de l'apoptose dans les cellules incubées) induit une nécrose massive des cellules LLC-PK1. L'induction de l'apoptose est également accompagnée d'une augmentation de la protéine pro-apoptotique Bax, résultant probablement de l'inhibition de sa dégradation par le protéasome.

Par ces démonstrations et contrairement à ce que l'on pensait initialement, il semble que les lysosomes jouent plutôt un rôle protecteur en séquestrant des quantités importantes d'aminoglycosides et c'est probablement uniquement après leur relocalisation dans le cytosol qu'ils sont capables d'induire de l'apoptose par activation de la voie mitochondriale.

SUMMARY

Gentamicin (GEN) belongs to the aminoglycoside antibiotics group. Aminoglycosides are used in hospitals to treat severe infections caused mainly by Gram negative bacteria. However, even if important efforts have been made to reduce their nephrotoxicity these three last decades, it remains of concern to clinicians and it has resulted in a marked limitation in the use of these otherwise potent and life-saving antibiotics. Aminoglycosides are eliminated by glomerular filtration and reabsorbed by the proximal tubular epithelial cells by receptor-mediated endocytosis. Once capture by megalin receptor, they are finally accumulated inside the lysosomes. The demonstration that these antibiotics are able to induce cell death by apoptosis in conditions mimicking clinical situations has brought new important elements for the identification of the mechanism underlying aminoglycosides nephrotoxicty.

The incubation of LLC-PK1 cells (pig renal proximal cell line) with gentamicin 1 to 3 mM for 0 to 72 hours resulted in induction of apoptosis which is time- and drug concentration-dependent. The first alteration observed in LLC-PK1 incubated with GEN is a lysosomal destabilization significant as from 2 hours of contact with the drug, followed by an increase of the cytosolic pro-apoptotic Bax protein (significant after 8 hours), known to activate mitochondrial pathway which was detected in GEN-induced apoptosis after 10 hours of incubation. The activation of the mitochondrial pathway allowed the release of cytochrome c, which has been observed after 12 hours in parallel to caspase-9 activation. Finally, caspase-3 was activated after 24 hours and in the same time, nuclear fragmentation (a morphological characteristic of apoptotic cell) was also detected.

The lysosomal destabilization could allow the release of lysosomal constituents able to induce apoptotic signalling such as cathepsins but also such as gentamicin itself. The involvement of gentamicin in apoptotic signalling induction from cytosolic compartment has been tested by taking advantages of the electroporation technique (delivery into the cytosol of impermeant drugs). This delivery of gentamicin inside the cell allows us to by-pass the lysosomal accumulation. The electroporation of low amount of gentamicin was sufficient to induces apoptosis (about 10-100 times inferior to those needed for incubated cells) whereas electroporation of higher doses of GEN (similar to those needed to induce apoptosis after incubation) induced a massive necrotic cell death of LLC-PK1. Electroporation of apoptogenic concentration of gentamicin has also been associated to an increase of Bax protein, resulting from a decrease of its degradation by the proteasome as suggested by the observation of an increase in ubiquitinated-Bax.

By those observations and at the opposite of our first though, lysosomes seems to be to be rather “protective” by sequestering high amount of toxic GEN, and it is only once released in the cytosol that they are able to induce proximal tubular epithelial cell death by apoptosis.